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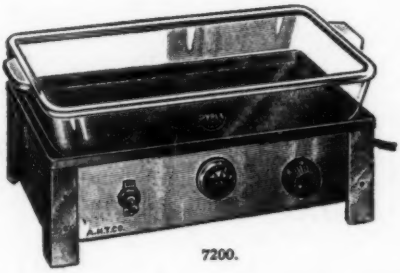

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STUDIES ON THE CARDIAC STOMACH OF THE STARFISH, ASTERIAS FORBESI [1]

JOHN MAXWELL ANDERSON

Department of Zoology, Cornell University, and Marine Biological Laboratory, Woods Hole, Mass.

The peculiar feeding methods of starfishes have attracted attention since at least the time of Aristotle. Problems involved in explaining the various phenomena of the feeding process have been investigated repeatedly, yet in many cases the solutions advanced for the problems are less than satisfactory. For example, "the old starfish-clam question," of the methods by which the starfish gains access to the interior of a bivalve, still awaits a convincing answer (see Reese, 1942). Also deserving of closer scrutiny is the mechanism by which food is brought into the digestive tract of the starfish. As Tiedemann (1816) points out, many of the ancients held the erroneous belief that the starfish ingested the soft parts of snails and bivalves by exerting suction. Müller (1788–1806?)[2] and Cuvier (1805) are cited by Tiedemann as the first to call attention to the characteristic eversion of the cardiac stomach of *Asterias*, a process which in its superficial aspects has been described many times in the years since Tiedemann's monograph appeared.

The general mechanism bringing about eversion of the cardiac stomach is now rather well understood, owing in part to Cuénot's account (1887); however, it is apparent that many features of the retraction of the stomach after feeding remain to be explained. The tough strands connecting the cardiac stomach with the ossicles in the floor of each ray have been variously referred to as mesenteries, as tendons, as strands of connective-tissue infiltrated by muscle fibers, and as retractor muscles, revealing a fundamental uncertainty as to their actual function. In addition, the relationships between these strands and the wall of the stomach have apparently never received an adequate description.

The present report is an attempt to interpret the results of a series of dissections and histological studies on the structure of the cardiac stomach of *Asterias forbesi*, particularly as these results are related to observations and preliminary physiological experiments on the mode of operation of the retractor system. Attention is also directed toward the relationship which exists between the distribution of retractor

[1] The studies on which this report is based received support from the Sarah Manning Sage and Dr. Solon P. Sackett Research Funds of the Department of Zoology, Cornell University.

[2] Tiedemann's reference is to Vol. IV, p. 13, of Müller's *Zoologia Danica*; he does not specify a date. I have assigned the dates of the only 4-volume edition which appeared prior to Tiedemann's *Anatomie* but have been unable to check with the original.

fibers and certain other specific structural features of the wall of the stomach. No attempt will be made to explore the subject of the digestive and absorptive functions of the cardiac stomach, although these functions are imperfectly understood and require further study.

Material and Methods

The animals used in these studies were vigorous specimens, ranging from 5 to 8 inches in over-all diameter. They were maintained in running sea water at Woods Hole, or in 15-gallon marine aquaria at Ithaca. The starfishes were fed periodically with pieces of the soft parts of the quahaug, *Venus mercenaria*, or with chunks of frozen whiting fillets, previously thawed. No difficulty was encountered in inducing the animals to feed, once they had become acclimated to conditions in the aquaria. It should be noted, however, that the starfishes generally ignored the living quahaugs kept with them in the tanks; only once during a period of several months was a quahaug successfully attacked and opened by one of my starfish.

Preparation and treatment of specimens for preliminary physiological experiments will be described below, as necessary, in connection with the observations. For histological work, the central part of the alimentary tract was exposed by removing from the living animal, relaxed by exposure to sea water containing added $MgSO_4$, the aboral walls of the disk and of the proximal parts of the arms. The pyloric stomach and rectal caeca were discarded, and the portion of the cardiac stomach pertaining to a single arm was carefully excised by radial incisions through the wall of the stomach, and by cutting across the peristome. The extended attachments of the retractor strands to the ambulacral ossicles in the floor of the arm were severed, and the portion of the stomach thus freed was transferred to a petri dish of sea water with added $MgSO_4$, for further relaxation. In this manner, the cardiac stomach of a single specimen could be removed in 5 approximately equal parts. Larger pieces could not be easily manipulated during the following operation, which consisted of carefully spreading and pinning out the segments of the stomach in small, wax-bottomed dishes containing a little sea water. These spread segments were studied from either the inner or the outer surface. Suspensions of india ink in sea water, placed dropwise on the inner surface, facilitated observation of the currents maintained by the ciliated (or flagellated) cells of the lining epithelium. Study of the outer surface permitted determination of the precise courses of the retractor fibers upon and within the wall of the stomach.

Following such gross observations, the sea water was decanted from the spread preparations, and the tissue, still pinned in place, was flooded with one or another histological fixative. After partial fixation had occurred, the pins were removed, and small, carefully oriented parts of the stomach were excised and transferred to fresh fixative. After imbedding in paraffin or in gelatin, these smaller parts were sectioned in planes parallel with, or perpendicular to, the plane of the mouth.

Serial sections 5 μ in thickness, of material fixed in Zenker-formol, were stained in a variety of ways for over-all orientation and to permit the differentiation of muscular, collagenous, and elastic fibers in the retractor mechanism. Most useful in this connection were Mallory's phosphotungstic acid hematoxylin, Krichesky's modification of the Mallory triple connective-tissue stain, van Gieson's picro-acid fuchsin collagen method, and the Taenzer-Unna acid-orcein method. Feulgen preparations were also employed, as well as the periodic acid-Schiff routine for the

demonstration of glycogen and other polysaccharide compounds. Dilute toluidine blue was used to localize metachromatic elements. To reveal the distribution of lipids, material fixed in Baker's formol-calcium and imbedded in gelatin was sectioned on the freezing microtome, colored with sudan black, and counterstained with carmalum.

One additional type of preparation was found useful to reinforce anatomical interpretations from gross dissections. From a starfish 6 inches in diameter, one arm was removed, together with a portion of the disk containing the part of the stomach attached to that arm. The aboral body wall was removed, and the pyloric stomach, pyloric caeca, and gonads discarded, along with the distal two-thirds of the arm. The remaining piece consisted of the floor of the proximal portion of the arm, the floor of a pie-shaped segment of the disk, and that part of the cardiac stomach attached to this piece by its complete retractor system and by the peristome. This preparation, designed to show the relationship between the normal, retracted cardiac stomach *in situ* and its retractor harness, was fixed in Zenker-formol. The tissue was subsequently decalcified for 48 hours in a 10% aqueous solution of disodium versenate,[3] dehydrated, and imbedded in paraffin. Serial cross-sections were prepared at 10 and 15 μ, and the sections were stained as in the case of the spread segments of the stomach described above.

Observations

A. The retractor system

As revealed by aboral dissection, or by a sagittal section of an arm, what might be termed the *extrinsic* part of the retractor mechanism of the cardiac stomach consists of 5 pairs of triangular, mesentery-like sheets, one pair in each arm, which connect the wall of the stomach with the ambulacral ossicles in the floor of the arm. One side of each triangle is formed by a strand originating distally in the floor of the arm, usually in the neighborhood of the 30th pair of ossicles. The other side is represented by a similar, but shorter, strand which originates from the lateral aspect of the first ambulacral ossicle. As shown in Figure 1, these strands appear as the thickened, free, upper borders of a long, low, triangular, mesentery-like sheet of tissue, which binds the strands to the series of ambulacral ossicles throughout their length; the extended attachments of this sheet to the ossicles form the base of the triangle.

Microscopically, the strands are found to consist chiefly of fibrous connective tissue, both collagenous and elastic, mingled with rather scanty muscle fibers. The sheet below the strands also contains collagenous fibers which run obliquely upward from the sheaths of the ossicles and interdigitate with the fibers of the strands. The strands and the fibrous sheet are enclosed by a fold of mesothelium, continuous with the peritoneal lining of the body wall.

[3] An organic chelating agent, the di-sodium salt of ethylene diamine tetra-acetic acid. It has been successfully used in the demineralization of tooth and bone in vertebrates (see, for example, Birge and Imhoff, 1952, and Freiman, 1954) but to my knowledge has not previously been applied to calcareous tissues in invertebrates. Its advantage over the classical mineral-acid decalcifying procedure lies in the fact that the versenes apparently do not destroy residual enzymatic activity and other characteristics of tissues, of interest in histochemical studies. The samples of disodium versenate employed in these investigations were generously supplied by the manufacturer, Bersworth Chemical Co., Framingham, Mass.

The apex of the triangle is the point at which the four fibrous strands in each arm are bound together by a transverse, band-like connection (Figs. 1 and 2). This general region will be referred to as the *nodule*. It is a tough, fibrous structure attached to the outer surface of the stomach by many radiating bundles of fibers. The nodule projects from the surface of the stomach, and when the stomach is fully retracted usually lies above the 10th to 12th ambulacral ossicles.

Each of the 5 nodules may be considered as forming a point of transition between the *extrinsic* retractor mechanism in the arms, just described, and the *intrinsic* part of the system, which lies upon and within the wall of the stomach. Passing through the nodular region, the extrinsic strands coalesce and then give rise to a

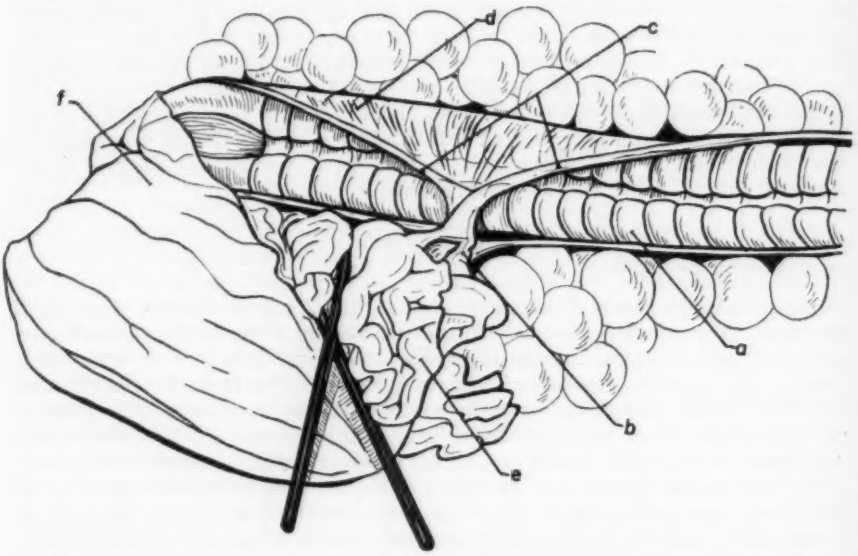

FIGURE 1. Dissection of an arm of *A. forbesi* to show extrinsic retractor mechanism. At *a*, ambulacral ossicles; *b*, region of the nodule; *c*, proximal and distal extrinsic strands; *d*, mesentery-like sheet; *e*, outer surface, and *f*, inner surface of a segment of the cardiac stomach, pinned aside.

complicated system of branching fibers radiating over the wall of the stomach. From each nodule arise two major bundles which proceed in opposite directions, in a plane generally parallelling that of the oral opening. Each of these major bundles gives off secondary branches, some of which are distributed over the roof of the cardiac stomach, but most of which proceed downward toward the mouth. These downward-coursing fibers branch in an apparently specific fashion, tapering gradually throughout their length. They enter the wall of the stomach, running under its peritoneal covering, and contribute fibers to its muscular and connective-tissue layers. Their ultimate branches may be traced, in sections, as far as the distinct line, above the mouth, which appears to mark the junction between stomach and

esophagus. The branching patterns of the terminal fibers coincide in a consistent manner with corresponding series of branching channels, to be described presently, which are characteristic of the lower portion of the wall of the stomach.

Histologically, the nodules and the intrinsic retractor strands may be described as variable mixtures of collagenous and muscular fibers, with an elastic fiber component throughout but concentrated mainly in the nodule and its major branches. The nodule appears as a band of complexly wound collagenous bundles, interspersed with smaller muscle fibers and bound together by branching strands of elastic tissue. In cross section, the large and moderate-sized bundles arising from the nodule appear to consist of a central, collagenous band, around which are arranged in a radial pattern 6 or 8 smaller, more compact bundles of collagenous fibers (Fig. 4). Aggregations of muscle fibers occupy the spaces between the collagenous strands, and the entire ensemble is enclosed by a mesothelial covering. In the

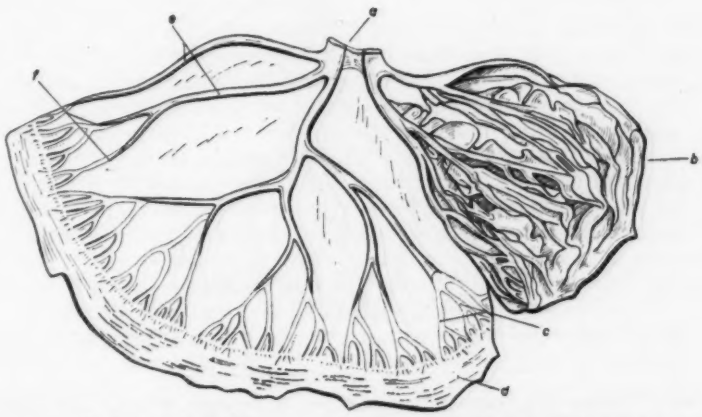

FIGURE 2. Excised and partially spread segment of cardiac stomach, as viewed from its outer surface. At *a*, region near the nodule; *b*, unspread portion of the stomach; *c*, branches of a channel system seen through wall of stomach; *d*, esophagus; *e*, intrinsic retractor fibers; *f*, point of entry of fibers into wall of stomach. As shown in the unspread portion at the right of the figure, the esophagus normally gathers the lower end of the stomach like a draw-string. It must be cut and stretched considerably to allow spreading of the stomach on a flat surface, as at the left.

smaller branches, farther removed from the nodule, the proportion of muscle fibers increases. These small branches eventually run under the peritoneal layer and often appear in sections as local thickenings of the outer coats of the stomach wall. They can be recognized, however, by the characteristic arrangement and direction of their fibers. Such a small branch appears as a central cord of collagenous tissue surrounded by larger or smaller groups of muscle fibers (Fig. 4, arrow).

B. The structure of the wall of the stomach

In its general histological composition, the wall of the cardiac stomach in the starfish is substantially as described for various genera, including *Asterias*, by

Hamann (1885), Cuénot (1887), Ludwig (1899), Schneider (1902), Chadwick (1923), and Hayashi (1935). There is considerable variation in details between various genera. In *Asterias,* the innermost layer is a pseudostratified, columnar epithelium composed of tall, slender cells interspersed with mucous gland cells and secretory cells containing coarse granules; the distribution of these glandular cells varies in different parts of the stomach. Between the elongated bases of the epithelial cells run the fibers of nerve cells constituting the "nerve layer" (Chadwick, 1923) or "neurofibrillar plexus" (Smith, 1937), varying in thickness in various regions. The extended basal processes of the epithelial cells rest upon, and often appear to intertwine with, the fibers of the connective-tissue layer lying just outside the neurofibrillar plexus. Beneath this collagenous and elastic coat (Fig. 4) are two layers of muscle fibers; the fibers of the inner layer take a generally circular

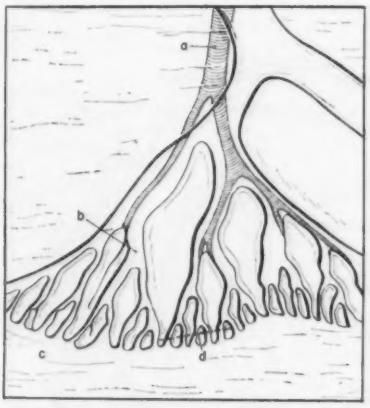

FIGURE 3. Portion of a channel system as viewed on the inner surface of the spread stomach. At *a*, intrinsic retractor bundle seen through the wall of the stomach; *b*, channels; *c*, esophagus; *d*, approximate plane of section shown in Figure 5. The eminences or ridges between the terminal branches of the channel patterns are covered with the closely packed specialized epithelial cells.

course, while those of the outer layer are longitudinal. The longitudinal muscle layer is covered externally by the visceral peritoneum, which, as elsewhere in the body, consists of a single layer of approximately cuboidal, flagellated cells. The connective-tissue layer appears to serve as the basement membrane of the epithelium and throughout its distribution sends short, branching fibers into the nerve layer and into the muscular layers. These fibers appear to be elastic strands which aid in maintaining the spatial relationships between the connective-tissue layer, the epithelium, and the muscular coats.

The most conspicuous structural feature of the cardiac stomach, viewed either in spread preparations (Figs. 2, 3) or in cross-sections (Fig. 5), is the series of branching systems of channels, or gutters, beginning at the line which marks the oral end of the cardiac stomach. The broad, widely-dispersed lower ends of these systems of grooves open on this line of demarcation; the gutters pass upward, con-

verging in a specific pattern which appears to follow generally the convergent paths of the retractor fibers described above. In the portion of the stomach pertaining to each arm there are usually 10 sets of these gutter systems. The smaller end-branches of the patterns, within the mouth, are deeper and narrower than the larger branches, higher in the stomach, formed by coalescence of the smaller channels. In its finer details, the distribution of the gutter patterns does not exactly follow the branching patterns of the retractor fibers, but there is a high degree of correspondence between the two systems.

Figure 3 shows a series of these gutter patterns as they appear on the inner surface of a spread segment of the cardiac stomach. The distribution of the related retractor fibers in the outer layers of the stomach wall is also indicated. Figure 5 shows a cross-section of the wall of the stomach through a portion of a single gutter system. It should be noted that the grooves follow a specific pattern, and that the alternating channels and ridges involve not the epithelium alone, but the entire thickness of the wall of the stomach.

As shown in Figure 6, the epithelium lining the bottom and sides of a single groove is composed of tall, slender, closely packed cells, each containing a small, ovoid nucleus. This kind of cell may be termed "typical" in appearance; that is, cells like these predominate in the epithelium of the cardiac stomach as a whole. Each of these typical cells bears a single, long flagellum with a single basal granule, and the distal ends of the cells are covered by a well developed brush border.

As Figure 7 shows, an entirely different kind of cell, which we may term a "specialized" cell, occurs on the ridges between adjacent grooves. Such a specialized cell is characterized by a thin, elongate nucleus occupying one-third to one-half of its length. The specialized cell bears more than one flagellum, the number varying between two and six. Each flagellum springs from a separate basal granule, and coarse, deeply staining fibrils from these granules coalesce to form a single strand which appears to join the nuclear membrane, or to proceed beside the nucleus toward the base of the cell. The elongated nuclei of the specialized cells are much denser and less granular than the ovoid nuclei of the typical cells; the Feulgen stain reveals the long nuclei as dense, homogeneous masses of DNA.

In addition to the specialized cells, the ridges between the channels bear many large mucous gland cells. The ovoid nuclei of these gland cells lie near the base of the epithelium, and the mucous goblets stream upward toward the free border, often compressed and distorted by the crowding of adjacent cells. At the lower end of each ridge there occurs a rather gradual transition to the type of epithelium characteristic of the esophagus; here the specialized cells are not so closely packed but are interspersed with very numerous mucous gland cells and cells filled with coarse, refringent granules.

A particularly sharp line of transition between the typical cells and the specialized cells occurs partway up the side of each ridge; this may be noted in Figure 5 and is shown more clearly in Figure 8. Below the line of transition, that is, nearer the bottom of the groove, there appears a band of typical cells which present interesting distal differentiations. These cells are distinguished by the fact that their distal ends contain masses or strands of small granules, lying about and below the basal granules of the flagella. The small granules appear to stream out of the cells of their origin and are scattered about in and on the brush border of neighboring cells. The general appearance of such a region of cells with distal differentiations

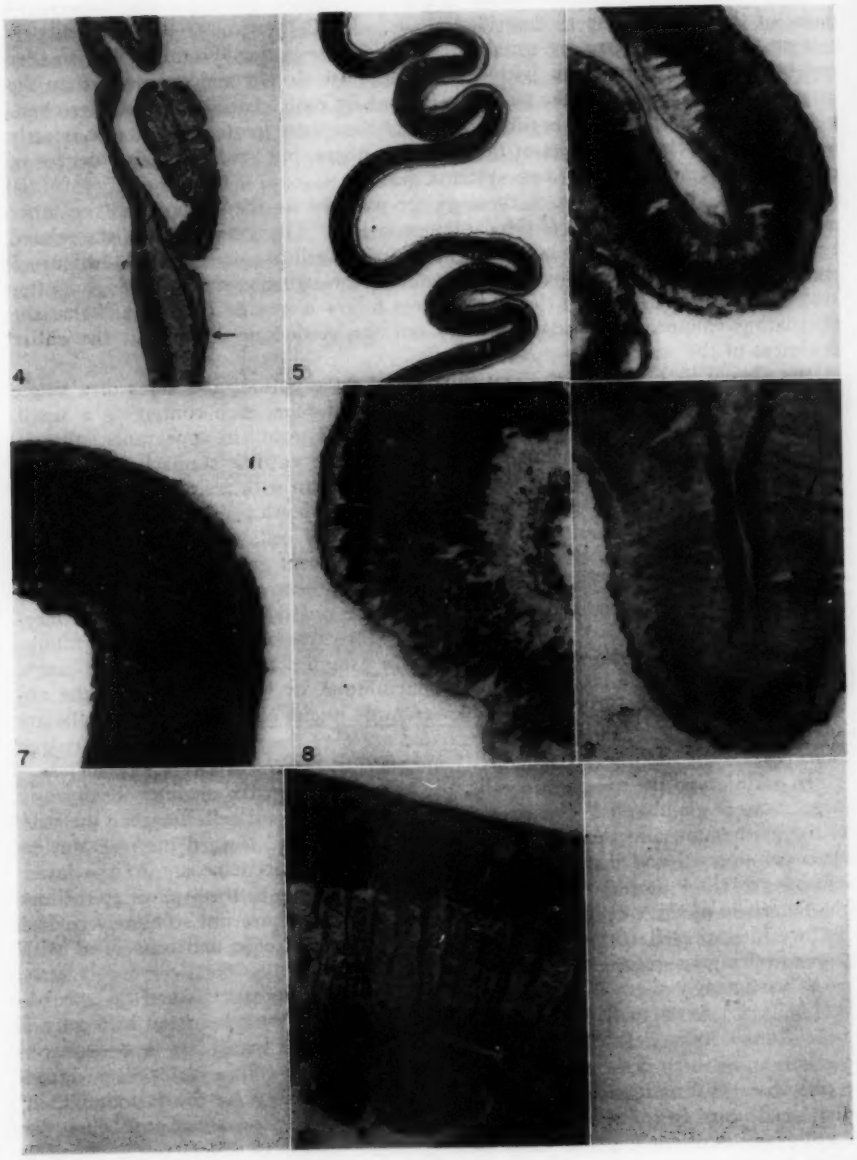

PLATE I

is shown in Figure 9. The small granules stain metachromatically with toluidine blue; they react positively with the periodic acid-Schiff routine, and this property is not destroyed by exposure to diastatic digestion. In addition, they stain deeply with acidulated orcein. In all respects except this last, they behave like the secretion of the mucous gland cells, but in their form and distribution they are very different from the mucous secretions. Small aggregations of similarly metachromatic and Schiff-positive secretion products occur in many of the typical cells throughout the lining of the cardiac stomach, but in the restricted, band-like areas of the walls of the grooves they are much more concentrated.

Figure 9 also shows the appearance of a type of gland cell not previously described. It occurs always in company with "typical" cells; its nucleus is located basally, and the shape of the cell is always like that of a flask with an extremely long and attenuated neck, extending to the free border of the epithelium. The finely-granular, dense cytoplasm of such cells stains deeply with phosphotungstic acid hematoxylin and with acidulated orcein, unlike ordinary mucous goblets. These cells may represent early stages in the secretory cycle of mucous gland cells.

The epithelium of the region below the ends of the groove patterns, which I have interpreted as corresponding to the esophagus, has a finely corrugated appearance. The corrugations (Fig. 3) involve only the epithelial and nervous layers, not the entire thickness of the wall. They are arranged in a circular direction, and this portion of the wall seems capable of considerable distension. The epithelium of the esophagus consists of specialized cells with long nuclei, like those covering the ridges at the lower end of the stomach. In this upper portion of the esophagus the specialized cells are accompanied by very numerous granular secretory cells and many mucous gland cells. There is a gradual transition to lower, more nearly

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PLATE I

With the exception of Figure 5, all figures in this plate are of material fixed in Zenker-formol, sectioned at 5 μ, and stained as indicated. Magnifications given are approximately those of the figures as they appear here, after enlargement and reduction.

FIGURE 4. Longitudinal section through upper region of stomach, showing a fairly large retractor fiber bundle in cross-section. Lumen to left. Mesothelial covering has been lost from outer surface of bundle. Note thickness of nerve layer; at arrow, small retractor fiber under peritoneum. Acidulated orcein, hematoxylin counterstain. 88 ×.

FIGURE 5. Frozen section, approximately 10 μ, through channel pattern at lower end of stomach (see Fig. 3, *d*). Lumen to left. Note contrast in epithelium in channels and on ridges. Sudan black, carmalum, after formol-calcium fixation. 52 ×.

FIGURE 6. Epithelium at base of channel. Note small, ovoid nuclei; brush border; basal granules and distal fibrils in cells. Light areas are mucous gland cells. Phosphotungstic acid hematoxylin. 278 ×.

FIGURE 7. Epithelium on ridge between channels; lumen to right. Note dense, elongate nuclei. Small clear areas are mucous gland cells. Phosphotungstic acid hematoxylin. 378 ×.

FIGURE 8. Transition zone between typical cells and specialized cells on the side of a ridge. Note multiple basal granules and distal fibrils in specialized cells; comma-shaped nuclei in these cells are not intact but have been cut at an angle. Phosphotungstic acid hematoxylin. 387 ×.

FIGURE 9. Typical cells with distal differentiations, in sides of a small channel. Note that similar areas in Figure 6 have not stained with hematoxylin. At arrow, flask-shaped gland cell. Acidulated orcein, no counterstain. 278 ×.

FIGURE 10. Epithelium at region of attachment of a retractor strand; *cf.* Figure 4. Note arcades formed by bundles of basal processes from epithelial cells; fibers and nuclei in nerve layer; absence of specialized cells from epithelium in this region. Lumen above. Phosphotungstic acid hematoxylin. 443 ×.

typical epithelial cells in the lower regions of the esophagus, and the secretory cells decrease in numbers.

The areas of the wall of the cardiac stomach lying between and above the orally directed groove patterns are covered by an epithelium which consists entirely of typical cells, interspersed with mucous gland cells. My studies indicate that the specialized cells with elongate nuclei are found nowhere in the wall of the cardiac stomach proper, except on the ridges separating the channels near the esophagus. The coarsely granular secretory cells appear never to occur in the cardiac stomach itself along with typical cells; they are found in the esophagus only, and only in company with specialized cells.

In regions adjacent to the attachment or insertion of retractor fibers, higher in the stomach, the nerve layer is often considerably thickened. In such areas (Figs. 1, 10), the epithelial cells send down bundles of elongate, fibrous basal processes which insert on the connective-tissue layer. These bundles form arcades through which course the fibers of nerve cells. Preliminary results of histochemical studies show that in well-fed animals droplets of lipid and deposits of glycogen are localized in the tapering portions of these epithelial cells, just above their fibrous processes.

C. Physiological studies

On the surface of the esophagus, on the ridges, and on the inner surface of the cardiac stomach generally, the currents produced by the vibratile organelles of the epithelial cells sweep in the direction from mouth to anus. Within the branching channels, however, the only currents I have been able to demonstrate are those which might be termed *centripetal*; that is, particles coming into a channel are whisked out of it again, toward the center of the digestive cavity.

Following the injection of 25 ml. of a 1:10,000 sea water solution of acetylcholine chloride into the perivisceral coelom of a large starfish, it is possible, by exerting pressure on the arms, to bring about the eversion of the cardiac stomach to any desired degree. Observation of the extruded vesicles of the cardiac stomach indicates that the major part of the "ballooning" of the stomach wall occurs in the region of the so-called esophagus. The region of the stomach marked by the channel systems appears to slide more or less straight downward from the mouth and may be protruded to a considerable degree. The channels at the lower ends of the systems may extend laterally a short distance over the lower, medial ends of the vesicles, but for the most part the channel systems lie on the inner faces of the vesicles, surrounding the narrow passageway leading upward into the stomach. It is likely that contributions to the stretched walls of the vesicles are made by the thin portions of the stomach wall lying between the separate sets of gutter systems.

The demonstration that acetylcholine facilitated the artificial eversion of the stomach, which cannot be caused at will in a normal, untreated animal, made it desirable to determine in a preliminary manner the effects of acetylcholine and of adrenalin on the various parts of the stomach involved in eversion and retraction. Accordingly, several starfishes were prepared by removal of the aboral wall of the disk and excision of the pyloric stomach and the roof of the cardiac stomach. In this way the inner surfaces of the cardiac stomach and of the esophagus were exposed, the closed mouth appearing at the center, below the esophageal area. The folds of the retracted stomach, together with the retractor harness, remained intact. A solution of acetylcholine in sea water (1:10,000), dripped slowly on the region

surrounding the mouth internally, caused a slow, steady, considerable enlargement of the oral opening. If the acetylcholine was flushed away with fresh sea water, and a 1:10,000 sea water solution of adrenalin chloride was dripped on the area around the open mouth, the oral opening immediately and rapidly closed. This alternate opening and closing of the mouth under the influence of the two drugs could be repeated many times, the solutions being washed away each time with fresh sea water. Little, if any, effect of either drug could be noted when solutions were applied to the folded walls of the retracted stomach, or to the retractor strands. Minute changes in the length of the extrinsic strands extending into the arms would be difficult to detect under these conditions; various small movements of the underlying body wall do occur, and these are reflected in small, apparently passive changes in the tension of the strands.

Discussion

Knowledge and understanding of the retractor mechanism of the cardiac stomach have had a slow growth and a varied history. Linck (1733; Appendix IV, p. 100, first commentary on Kade's para. IX) makes an indirect reference to 5 connecting ligaments in relation to the stomach. Cuvier, writing about the year 1800, in his description of the nervous system of the starfish, writes as follows (Ross' translation, 1802, Vol. II, p. 369): "Round the oesophagus we observe a girth of a soft whitish substance, which produces ten filaments, two to each of the branches . . . the two filaments belonging to each branch having arrived at the base of the osseous and articulated stalk . . . unite to form a short cord, which extends directly from the one to the other; . . . At the place where they are united, each produces a fasciculus of filaments, which are distributed to the stomach. . . . The appearance of all these filaments is rather tendinous than nervous, and that circumstance has hitherto chiefly prevented us from forming a decided opinion of their nature." [4] From this description, one is struck by the accuracy of Cuvier's observations; he is clearly describing the intrinsic and extrinsic retractor mechanisms of the cardiac stomach. And, obviously, he was not convinced that these filaments represented the nervous system, although he discusses them in connection with the nervous system and does not suggest for them any alternative function.

With less caution, Spix (1809), a disciple of Cuvier, gives a less accurate description of the retractor system of the stomach in *Asterias rubens*, stating that the nodules (Spix's own term: p. 440) on the wall of the stomach are ganglia, and that the various fibers and strands radiating from them are other parts of the nervous system.

Tiedemann (1816) corrects Spix's (and Cuvier's) error [5] and describes the

[4] Cuvier's original statement (T. II, p. 360) is as follows: *Autour l'oesophage s'observe une ceinture de substance molle et blanchâtre, d'ou partent dix filets: deux pour chacune des branches. . . . Les deux filets qui appartiennent à chaque branche étant arrivés à la base de la tige osseuse et articulee . . . se réunissent par un cordon court qui se rend directement de l'un à l'autre. . . . A l'endroit où ils se réunissent, part de chacun d'eux un faisceau de filets qui se distribuent sur l'estomac. . . . L'aspect de tous ces filets est plutôt tendineux que nerveux, et c'est sur-tout cela qui nous empêche de nous décider encore.*

[5] It is somewhat puzzling to note that Tiedemann, who was an enthusiastic follower of Cuvier and familiar with his work, does not mention Cuvier's suggestion of the nervous nature of these fibers, although in an extended footnote he practically ridicules Spix for his similar interpretation.

counterpart of the fibrous system in *Astropecten aurantiacus*, where it differs in many respects from that of *Asterias*. (The differences are in part presumably related to differences in feeding habits: whereas *Asterias* carries on much of its digestive activity with the vesicles of the cardiac stomach everted, *Astropecten* apparently everts the stomach only to engulf food which is then "swallowed" by retraction of the vesicles; see, for example, Cuénot, 1891, p. 416; Ludwig, 1899, p. 733.) Tiedemann, although he knew well the eversible nature of the cardiac stomach, describes the fibrous strands as "tendinous" (*sehnenartige*) and does not impute to them any retractor functions. He says merely (p. 43): "All of these strands connect the outer surface of the stomach with the inner surface of the skin, and hold the stomach spread and suspended in its place." [6]

Since the descriptions furnished by Cuvier and Spix, the pertinent literature reveals a surprising lack of interest on the part of investigators in what I have termed the intrinsic retractor mechanism. Ludwig (1899, p. 587) speaks of paired *Magenmesenterien*, arising from several roots on the outer surface of the stomach; here, however, as in most cases, the author deals almost exclusively with the nature and disposition of the extrinsic strands. With regard to these extrinsic parts, an interesting shift of opinion has occurred: their function as muscular retractor strands has been emphasized, while their fibrous nature, and the possibility that they may serve simply as ligamentous attachments, have been lost sight of. Hoffmann (1872, p. 6) writes of *Asterias rubens*: "The 10 flat bands which arise from the under surface of the stomach, run in pairs into the arms, and serve for the attachment of the stomach, consist of connective-tissue bundles infiltrated by muscle strands." [7] Cuénot (1887, p. 41) attributes the withdrawal of the stomach after feeding to the contraction of muscles in the mesenteric strands (". . . *pour le réintégrer, les muscles des brides mésentériques se contractent, et l'attirent à l'intérieur*"). Cuénot's 1887 opinion is essentially restated in his last work on this subject (1948). Ludwig describes the extrinsic retractors as mesenteries and states clearly that they are composed of connective-tissue fibers, with only a slight representation of muscular tissue. Chadwick, in his monograph on *Asterias* (1923), shows by a figure (Plate V, Fig. 43) that he had observed the true appearance of the triangular extrinsic sheets, with the nodules at their apices; however, he considers the strands as retractor *muscles* and gives no indication of their composition, or of their possible function as simple attachments for the stomach. He writes (p. 20): "The stomach is furnished with five pairs of retractor muscles. . . . These muscles come into action when the cardiac sac is retracted after eversion. . . ." Modern British and American textbook authors unanimously follow Chadwick and speak of the strands as retractor muscles, which by their contraction withdraw the stomach after feeding.

As the earlier authors were aware, and as indicated by the present study, the histological structure and the anatomical relationships of the extrinsic strands and sheets do not appear to justify this interpretation. Each of the nodules on the

[6] Tiedemann's statement (p. 43): *Alle diese Faden verbinden die äussere Fläche des Magens mit der inneren Fläche der Haut, und erhalten den Magen ausgebreitet und aufgehängt in seiner Lage.*

[7] Hoffmann writes (p. 6): *Die 10 platter Bänder welche von der unteren Fläche des Magens entspringen, paarig in die Armen verlaufen und zur Befestigung des Magens dienen, bestehen aus mit Muskelfasern durchsetzten Bindegewebsbündeln.*

wall of the stomach is bound to the ambulacral ossicles of its arm by 4 tough, collagenous strands, which are further secured by the mesentery-like sheets. Comparison of a series of preserved specimens with stomachs in different stages of eversion or retraction reveals that the nodules do move in relation to the ossicles, but only very slightly; in the most strongly everted specimen the nodules lay over the 4th and 5th ossicles. These facts, and the very scanty musculature of the extrinsic parts of the system, make it clear that the principal function of the extrinsic strands and sheets is to provide a firm but elastic and slightly movable anchorage for the nodules, and through the nodules, for the intrinsic retractor fibers and the wall of the stomach itself. Against these 5 anchored points the branching and widely distributed strands of the intrinsic retractor system may passively stretch and pull, during eversion, as the mouth opens and the increased intracoelomic pressure inflates and everts the thin-walled pockets of the stomach.

The fact that the nodules do move during eversion, and that the extrinsic strands do contain muscle fibers, suggests that these parts possess a slight contractility useful in returning the nodules to their normal positions at retraction. The more significant aspects of retraction, however, involving the deflation and withdrawal of the vesicles and the folding of the walls of the stomach into their positions within the disk, undoubtedly depend upon the contractility and elasticity of the intrinsic retractor mechanism. In the constituent strands of this system, as in the wall of the stomach itself, muscular fibers are well developed. The proximal ends of these fibers are ultimately attached to the relatively fixed nodules; their distal ends insert on the walls of the cardiac stomach in widely distributed patterns near the esophagus. Contraction of these fibers would therefore "pucker" the walls of the everted vesicles, reducing their volume and forcing the return of their fluid contents into the perivisceral coelomic cavities of the arms. The specificity and constancy of the patterns of distribution of the intrinsic retractor fibers make it apparent that the folding of the wall of the stomach is not a random process brought about by traction from the remote extrinsic parts, but occurs in an orderly, specific fashion, controlled by the intrinsic musculature.

The mouth is opened by relaxation of the fibers of the relatively thick circular muscle layer in this area, and contraction of the longitudinal muscle fibers, which in this region (peristome, *Mundhaut*) take a radial course. Cuvier (1837, T. V., p. 377) recognizes the reciprocal functions of the circular and longitudinal muscular layers in the control of the mouth, remarking that the opening and closing of the mouth resembles that of a pupil. The antagonistic effects of acetylcholine and adrenalin in controlling these processes may be assumed to be operative in the normal feeding activities. Previous investigators have demonstrated that muscular tissues of the alimentary tract in *Asterias* contain appreciable quantities of acetylcholine (Bacq, 1947) and exhibit marked cholinesterase activity (Augustinsson, 1946). The results of the present preliminary studies indicate that these chemical mediators operate in relation to specific processes involved in the normal functions of the alimentary system. It would be helpful to know whether the antagonistic effects of acetylcholine and adrenalin on the muscles of the oral region reflect reciprocal innervation of the circular and longitudinal muscle layers of the peristome from separate components of the nervous system. The details of the innervation of these muscular layers in *Asterias* are apparently unknown. Hamann (1885) describes very generally the origin of the continuous peristomial and visceral nerve

plexus layer from the nerve ring bordering the peristome. The relationships of the visceral nerve layer to the other components of the "ectoneural system" are outlined and discussed by Ludwig (1899, pp. 547 *ff.*). Smith (1937) describes superficially the probable sensory relations of the gut wall, but the presence of specific sensory and motor pathways in the nerve layer of the alimentary tract has not been demonstrated. It is to be hoped that the elegant researches of Smith (1937, 1950) on the sensory-neuro-muscular aspects of the somatic parts of the starfish may soon be extended to elucidate the functional details of the visceral system.

Whatever the related facts of innervation and neurohumoral activation may be, and granting readily the preliminary and tentative nature of the physiological conclusions drawn from the present studies, the following resumé of events in eversion and retraction of the cardiac stomach seems justified on the basis of observations to date:

Eversion: (a) opening of mouth (cholinergic fiber activity?); relaxation of muscles in wall of stomach and in intrinsic retractor strands;
(b) contraction of muscles in body wall, increasing intracoelomic pressure against relaxed walls of the stomach, which are forced out through mouth as fluid-filled vesicles.

Retraction: (a) relaxation of muscles in body wall, decreasing intracoelomic pressure;
(b) contraction of muscles in wall of stomach and in retractor system, forcing coelomic fluid back into cavities of arms, deflating vesicles, and folding walls of stomach into normal internal positions; closing of mouth (adrenergic fiber activity?).

If one assumes a degree of elasticity in the body wall, relaxation of the body wall musculature would permit an active increase in the volume of the perivisceral coelom and thus by elastic recoil exert a certain amount of suction on the vesicles of the everted stomach. Whether or not this actually occurs, it could theoretically operate as an additional factor in the retraction of the stomach.

The significance of the specific and conspicuous patterns of channels on the wall of the cardiac stomach remains to be explained. It is surprising that they have apparently escaped description by previous observers, who have contented themselves by remarking generally on the folded nature of the walls of the retracted stomach. The oversight may be accounted for by the fact that the patterns are unsuspected when one examines the retracted stomach. Under normal circumstances, the investigator rarely sees a strongly everted stomach; the "humped" position of the body during feeding effectively conceals the details of the everted vesicles. They may be properly examined only when the cardiac stomach is removed from the animal and spread. At first glance, one would expect that such convergent grooves, leading upward from the esophagus into the stomach, would be of value in providing protected channels for upward-flowing currents. However, as experiments with india-ink suspensions indicate, the channels are the only places on the surface of the epithelium where currents do *not* flow upward.

The specialized cells clothing the ridges between these channels may be noteworthy in this connection. Such cells have never been described in the classical histological studies of the cardiac stomach. Smith (1937), in a report dealing primarily with the nervous system, noted similar cells in the cardiac stomach of

Marthasterias glacialis. He does not clearly describe their distribution, stating merely that they are to be found only in those parts of the stomach where there is developed an extensive neurofibrillar plexus. Admitting the lack of direct evidence, Smith nevertheless suggests that these cells with "cigar-shaped nuclei" may represent sensory receptor cells. "Their presence would, indeed, not be unsuspected in an evaginable part of the gut which is capable of being wrapped round the prey during the process of ingestion" (p. 146). In the stomach itself, as the present study shows, these specialized cells are wholly restricted to the regions of the gutter systems; they are found otherwise only in the epithelium of the esophagus, which appears to contribute very significantly to the evaginated vesicles. These cells show no evidence of secretory activity, and, aside from their peculiar nuclei, their only other distinctions from typical cells (*i.e.*, multiple flagella, with well developed intracellular fibrillar connections) might well be adaptations to sensory receptor functions. It may be suggested that the alternating grooves and ridges, which are fundamentally of the nature of folds in the stomach wall, may have developed in conjunction with the distribution patterns of the terminal fibers of the intrinsic retractor system, and in response to their folding actions. The specialized cells may simply form patches of sensory epithelium between the grooves. One might emphasize also the fact that although the regions bearing the grooves and their supposed sensory epithelial patches are not themselves strongly everted at feeding, they do form an almost continuous lining for the narrow passageway leading upward into the stomach, through which all ingested material must pass.

The distal differentiations of the otherwise typical cells in the side walls of the channels remain without explanation. The granules which they produce are apparently some type of secretion showing similarities to mucus, but differing in the details of their formation and appearance. The cells are always found in such positions that their secretions will be carried outward, away from the surface of the epithelium, by the centripetal currents produced in the channels. It is to be hoped that further studies of these cells, and of the nature of their secretion product, will clarify their functions.

Summary and Conclusions

Studies on the structure and functions of the cardiac stomach and its retractor mechanism reveal that the outer surface of the stomach is provided with a complex, specifically distributed system of tapering, branching fibers of muscle and connective tissue, inserting ultimately on the muscular and connective-tissue coats of the stomach wall. The origins of these fibers are from 5 band-like nodules, the points of attachment to the stomach of the paired retractor strands in the arms. These strands, the *extrinsic* parts of the retractor system, form the upper borders of triangular, mesentery-like sheets which bind them to the ambulacral ossicles throughout their length. The extrinsic parts, as reported by many early investigators, consist chiefly of collagenous fibers and contain very scanty musculature; they appear to move but little in eversion or retraction. It is suggested that they serve merely to anchor the 5 nodular regions of the stomach, against which the branching *intrinsic* retractor fibers of the wall of the stomach may stretch and pull at eversion. It is further suggested that the more significant aspects of retraction involve not the apparently very slightly contractile extrinsic parts of the system, but the widely distributed, muscular branches of the intrinsic mechanism. This interpretation is

not in accordance with that of modern authors, who refer to the extrinsic strands as retractor *muscles* and disregard the intrinsic parts of the system completely. A brief summary of the development of this narrow emphasis is presented.

The terminal branches of the intrinsic fibers form patterns coinciding with a series of branching systems of channels on the inner surface of the stomach. These gutters open at the line of junction between stomach and esophagus and run upward, coalescing to form larger and broader channels higher in the stomach. The bottoms of these channels are lined by typical, non-specialized, tall epithelial cells, each with a small, ovoid nucleus and bearing a single flagellum. The ridges between adjacent grooves are covered by specialized cells, each with a long, spindle-shaped nucleus and bearing from two to six flagella. It is not unlikely that, as Smith has suggested, these cells represent sensory receptor elements. They are localized in a part of the stomach which, not itself strongly everted into the vesicles, forms the lining of the narrow gullet through which all ingested food must pass. Similar cells are found in the lining of the esophagus, which appears to form the major share of the vesicles of the everted stomach. The specialized cells are not found in the stomach above the lower parts of the channel systems.

Although the currents maintained over the lining of the stomach generally sweep from mouth to anus, within the gutter patterns the flagella whisk particles not upward, but outward into the lumen. Certain secretory cells localized on the walls of the gutters may contribute their secretions to these centripetal currents; their function is unknown.

Acetylcholine and adrenalin act antagonistically in controlling the opening and closing of the mouth. Acetylcholine opens the mouth by causing contraction of the longitudinal (radial) musculature of the peristome and esophagus; adrenalin reverses this action by bringing the circular muscles into contraction. It is tentatively suggested that these effects may reflect reciprocal innervation of these muscular layers from different components of the circumoral nerve ring. No action of chemical mediators has been demonstrated on the elements of the retractor systems, extrinsic or intrinsic.

LITERATURE CITED

Augustinsson, K. B., 1946. Cholinesterase in some marine invertebrates. *Acta Physiol. Scand.*, **11**: 141–150.

Bacq, Z. M., 1947. L'acétylcholine et l'adrénalin chez les invertébrés. *Biol. Rev.*, **22**: 73–93.

Birge, E. A., and C. E. Imhoff, 1952. Versenate as a decalcifying agent for bone. *Amer. J. Clin. Path.*, **22**: 192–193.

Chadwick, H. C., 1923. Asterias. L. M. B. C. Memoir XXV, University Press, Liverpool.

Cuénot, L., 1887. Contribution à l'étude anatomique des Astérides. *Arch. Zool. exp. et gén., Sér. 2*, T. 5 *bis*, Supp., Mém. 2: 1–144.

Cuénot, L., 1891. Études morphologiques sur les Échinodermes. *Arch. Biol.*, **11**: 313–680.

Cuénot, L., 1948. Embranchement des Échinodermes. *In* Traité de Zoologie, ed. P.-P. Grassé, T. XI. Masson, Paris.

Cuvier, G., 1805 [8]. Leçons d'anatomie comparée, T. II and III. Crochard, Paris.

Cuvier, G., 1837. Leçons d'anatomie comparée, 2nd Ed., T. V. Crochard, Paris.

Freiman, D. G., 1954. Organic chelating agent in the demineralization of bone for histochemical study of alkaline phosphatase. *Amer. J. Clin. Path.*, **24**: 227–231.

Hamann, O., 1885. Beiträge zur Histologie der Echinodermen. Heft 2. Die Asteriden anatomisch und histologisch untersucht. Fischer, Jena.

[8] Appeared originally in 1800; the copy to which I have had access is a re-issue of the first edition, dated 1805.

HAYASHI, R., 1935. Studies on the morphology of Japanese sea-stars, I. Anatomy of *Henricia sanguinolenta* var. *ohshimai*, n. var. *J. Fac. Sci., Hokkaido Imp. Univ., Ser. VI, Zool.*, **4**: 1–26.

HOFFMANN, C. K., 1872. Zur Anatomie der Asteriden. *Niederl. Arch. f. Zool.*, **2**: 1–32.

LINCK, J. H., 1733. De stellis marinis, Appendix IV: Kade, Stellae marinae, quinque radiorum holsaticae coloris violacei, Anatome. Schusterum, Lipsiae.

LUDWIG, H. (WITH O. HAMANN), 1899. Echinodermen, II. Buch. Die Seesterne. *In* Klassen u. Ordnungen des Thierreichs, Bd. 2, Abt. 3. Ed. H. G. Bronn. Winter, Leipzig.

MÜLLER, O. F., 1788–1806. Zoologia Danica, 3rd Ed., Vol. 4, p. 13. Havniae. (Cited from Tiedemann; original not available to me.)

REESE, A. M., 1942. The old starfish-clam question. *Science*, **96**: 513.

ROSS, W., 1802. Cuvier's lectures on comparative anatomy. English translation of 1st edition. Vol. 2. Wilson, London.

SCHNEIDER, K. C., 1902. Lehrbuch der vergleichenden Histologie der Tiere. Fischer, Jena.

SMITH, J. E., 1937. On the nervous system of the starfish, *Marthasterias glacialis* (L). *Phil. Trans. Roy. Soc., B*, **227**: 111–173.

SMITH, J. E., 1950. Some observations on the nervous mechanisms underlying the behavior of starfishes. *Symp. Soc. Exp. Biol.*, **IV**: 196–220. Academic Press, New York.

SPIX, J. B. VON, 1809. Mémoire pour servir à l'histoire de l'astérie rouge, *Asterias rubens*, Linn.; de l'actinie coriacée, *Actinia coriacea*, Cuv.; et de l'alcyon exos. *Ann. Mus. Hist. Nat., Paris*, **13**: 438–459.

TIEDEMANN, F., 1816. Anatomie der Röhrenholothurie, des pommeranzfarbigen Seesterne, und Stein-Seeigels. Thomann, Landshut.

THE RHYTHMIC ACTIVITY OF THE QUAHOG, VENUS MERCENARIA, AND ITS MODIFICATION BY LIGHT [1, 2]

MIRIAM F. BENNETT [3]

Department of Biological Sciences, Northwestern University, Evanston, Ill., and the Marine Biological Laboratory, Woods Hole, Mass.

The correlation of activities of animals in their natural environments with specific times of day, tide, and phases of the moon is an often-observed phenomenon. Also, it is a well-established fact that some of these activities, especially those correlated with the day-night cycle, persist even after the organism has been placed under conditions in which all factors known to stimulate the organism are kept constant (see reviews: Welsh, 1938; Park, 1940; Kleitman, 1949; and Webb, 1950). The cycles of activity which continue unaltered under constant conditions have been spoken of as endogenous or persistent rhythms as opposed to exogenous ones which continue only while the particular activating stimuli are being received by the organism. It has been suggested very strongly that the so-called endogenous rhythms are dependent on highly stable, inherent, physiological mechanisms or "internal clocks." However, the possibility that the animals that display persistent cycles of activity are actually reacting to some cyclically occurring physical forces of the external environment, in a manner not yet determined, has also been postulated by investigators.

Early in the present century, persisting or endogenous tidal cycles of activity were reported for certain marine animals. These papers have been reviewed by Brown, Fingerman, Sandeen and Webb (1953) who have demonstrated the existence of a persisting tidal rhythm superimposed upon the diurnal rhythm of color change in the fiddler crab, *Uca pugnax*. Moreover, these investigators have shown that in these crabs a semi-lunar or 14.8-day cycle of color change is the result of the precise relationship between the diurnal and tidal rhythms. More recent work illustrates that the rates of O_2-consumption of these crabs also reflect the presence of these rhythms (Brown, Bennett and Webb, 1954). Here it was found that the rate of O_2-consumption was generally greatest, other factors being equal, one to two hours before the times of low tides in the area from which the animals were collected. Again, it was clearly evident that the systematic moving of the tidal influence relative to the diurnal one produced characteristic patterns of O_2-consumption which recurred about every 15 days, or once every semi-lunar period. There was

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[3] Present address: Department of Biology, Sweet Briar College, Sweet Briar, Virginia.

also a suggestion in the results of these studies that another cycle of O_2-consumption, a lunar or 29-day one, was present for this species.

The O_2-consumption rates of certain of the marine molluscs have also been correlated with environmental factors. *Patella, Mytilus,* and *Haliotis* were reported to have persisting rhythms of O_2-consumption under laboratory conditions (Gompel, 1937). Although the hourly data of these studies varied considerably, the highest rates of O_2-consumption were generally apparent about the times of high tides and lower rates were recorded at the times of low tides. More spectacular rhythms of O_2-consumption are described by Sandeen, Stephens and Brown (1954) for the marine gastropods, *Littorina littorea* and *Urosalpinx cinereus.*

Other molluscs exhibit tidal rhythms of motor activity when maintained under constant conditions. Bohn (1904) reported that *Littorina rudis* became active at 15-day intervals in the laboratory. In its natural habitat this snail is covered by water only during the semi-lunar high, high tides which normally occur about every 15 days. A persistent rhythm of locomotory activity related to the tides is displayed by *Nassa obsoleta* (Stephens, Sandeen and Webb, 1953). Also, the filtering rate of species of *Mytilus,* using colloidal graphite, varies rhythmically in phase with the tidal cycles in the area from which the mussels were collected (Rao, 1954).

Some of the characteristics of these persistent rhythms have been elucidated by various experimental procedures of which light and temperature modifications have been employed most often. The effects of changes in light on the diurnal rhythm of color change for fiddler crabs are discussed by Brown and Webb (1949), Webb (1950), Brown and Hines (1952), and Brown, Fingerman and Hines (1954). Brown and Stephens (1951) have reported modifications of this cycle which were induced by photoperiod. The only paper to date which describes the experimental modification of a tidal rhythm by changes in illumination is that of Brown, Fingerman, Sandeen and Webb (1953). These investigators found that when the diurnal color change rhythm for *Uca pugnax* is shifted by an appropriate light stimulus, the tidal rhythm is shifted correspondingly. Temperature studies have demonstrated that the frequencies of both the diurnal and tidal rhythms of color change for *Uca* are temperature-independent over fairly great ranges, at least 6° C. to 26° C. for the diurnal cycle (Brown and Webb, 1948), and at least 13° C. to 30° C. for the tidal cycle (Brown, Webb, Bennett and Sandeen, 1954). The tidal cycle of pumping for *Mytilus* is independent of temperature in the range 9° C. to 20° C. (Rao, 1954).

The remarkable stability of the so-called endogenous rhythms has supported the hypothesis that the possession of these cycles is of adaptive significance for these animals. Thus, they are able to anticipate the time of day or tide, or phases of the moon. Organisms whose feeding activity and/or breeding periods seem to be correlated with specific times in these cosmic cycles would appear to be likely subjects for the study of biological rhythms.

Korringa (1947) was able to show that the time of spawning of oysters and several other lamellibranchs exhibits a lunar periodicity. The results of studies on oysters (Korringa, 1952) and quahogs (Loosanoff, 1939) in their natural habitats, however, have been interpreted as indicating that these animals do not display any persisting activity correlated with the time of day or tide.

It was the purpose of the present study, therefore, to determine whether or not the common quahog, *Venus mercenaria,* an animal of the intertidal zone and shallow

water, exhibits activity cycles under constant laboratory conditions. The activity rhythms that were observed have been related to the day-night cycle, to the phases of the tidal cycles, and hence to lunar periodicity. Also, an attempt was made to modify the character, or phase relationships to time of day, of the cycles of activity by light changes.

Material and Methods

The activities of three groups of quahogs were studied in Evanston, Illinois, during the winter and spring of 1953. They were obtained from a local fish market, and had been collected originally in the Chesapeake Bay region of Virginia. A fourth group was studied during June, 1953; these were clams which had been collected in Little Harbor, Woods Hole, Massachusetts. In December, 1953, the activities of two more groups of quahogs were studied concurrently in an attempt to ascertain the effects of light changes on the persistent cycles of activity. These animals again were obtained from a local market in Evanston, and had been collected in Virginia.

All the clams used for the activity studies were adults. There were eight to ten animals in each group; additional clams, which were obtained at the same time as those whose activity was being recorded, were kept under the same conditions as those that were being studied. In the event that a clam died, one of the additional animals was substituted, and the activity records of the original animal were discarded. Over the course of the observations, the mortality rate was very low. The great majority of the quahogs were in good condition even after having been in the laboratory for as long as four weeks.

The quahogs were placed in finger bowls containing sea-water which was changed at irregular intervals so that any observed rhythms could not be attributed to a regular time of water change. During the course of the study of the first four groups, the illumination at the surface of the water, as measured with a Weston photometer, was constant at approximately 2 ft. c. The source of illumination was fluorescent lights. The temperature of the water varied irregularly between 20° C. and 22° C.

Steel wires were bent to fit snugly over one of the valves of each animal from umbo to ventral edge. To these wires were tied threads which, in turn, were connected to sensitive heart levers or to the levers of the automatic recording spring scales described by Brown (1954). These levers recorded the opening and closing movements of the clams continuously on slow kymographs for periods of two to three weeks.

The daily record of each clam was analyzed by determining the times of opening and closing and the duration of the period open or active. The results obtained from the eight to ten animals of a group were averaged to obtain values for the total amount of time open per day. The time open was plotted against the days.

The following procedure was followed in the study of the effects of changes of illumination on the rhythms. One group of animals, the control group, was maintained under constant illumination of 2 ft. c. The experimental group was kept in a photographic dark room. For five consecutive nights (8 P.M. to 8 A.M.) this group was exposed to fluorescent lights providing an intensity of 100 ft. c. During the day (8 A.M. to 8 P.M.) they were kept in darkness. From 8 P.M. of the last day of darkness until the end of the experimental period, they were maintained at

an illumination of 2 ft. c. During the three-week period, the temperatures of the two rooms in which the clams were kept were recorded continuously on Taylor thermographs, and were seen to fluctuate between 20° C. and 22° C. The records for these animals were analyzed as were those for the clams of the first four groups. However, the average values for three consecutive days were averaged and this average used as the value for the second of the three days, *e.g.*, the figures for December 4, 5, and 6 were averaged and the resulting value used as the time open for December 5. Such procedure was used to obtain the relationship illustrated in Figure 5, and was thought to render the comparisons between the experimental and control curves more significant.

The form of the average diurnal curve for each group was ascertained by averaging the times open or active for each of the 24 daily, one-hour periods of 15 consecutive days. These 24 values were converted further to the percentage of 60 minutes open or active. All phases of a possible tidal influence are represented in

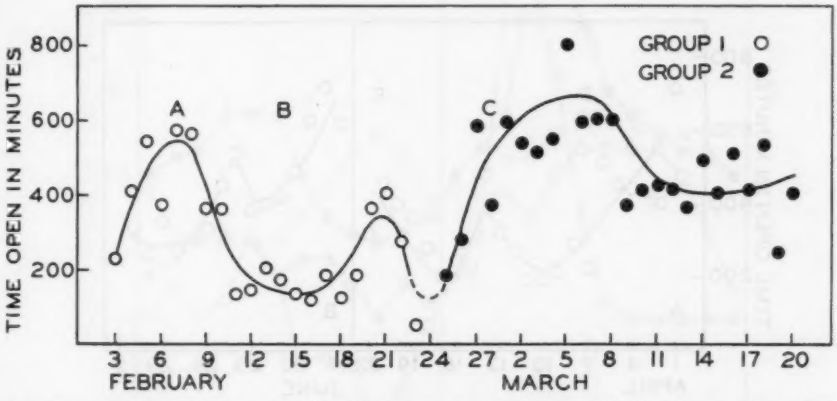


FIGURE 1. The long-term cycles of activity for group 1 (open circles) and group 2 (black circles) which were studied in Evanston, Ill. For explanation of A, B, and C, see text.

each of the values, and are thereby randomized since data for 15 days were used. In the cases in which the particular observational period was longer than 15 days, a 15-day period was arbitrarily selected, usually from the middle of the period.

Conversely, the diurnal rhythm was rendered random to reveal the character of the tidal rhythm in the following manner. The values for activity of the 24 daily, one-hour periods for each of 15 successive days were moved backwards at an average rate of 50 minutes a day, *i.e.*, on succeeding days later one-hour periods became aligned with earlier ones. By this means any possible tidal cycles were kept in phase with one another in the vertical columns of data. By continuing this process, repeating with the data of the first 14 days until a total of 29 days had been included, every phase of a diurnal influence was moved across the whole 24-hour period. Again, each of the 24 values has been expressed as the percentage of 60 minutes active or open. In Figure 4 the percentage of each hour open was plotted against time in hours in such a manner that the first minimal point fell between the sixth

and seventh hours. This procedure was obviously perfectly legitimate since the points on the abscissa merely indicated consecutive hourly periods, and do not represent actual times of any given day.

Results

1. The studies of animals maintained under constant conditions

A. The long-cycle rhythm

The average time open in minutes per day was determined for each group of quahogs. Long-cycle rhythms of activity were rendered clearly evident by plotting the time open (ordinate) against the date (abscissa). These cycles of activity for the four groups of animals are illustrated in Figures 1 and 2. It can be seen in the curve for groups 1 and 2 (Fig. 1) that major peaks of activity occur about 29 days apart and a minor peak occurs about half way between. The curve for group 3 (Fig.

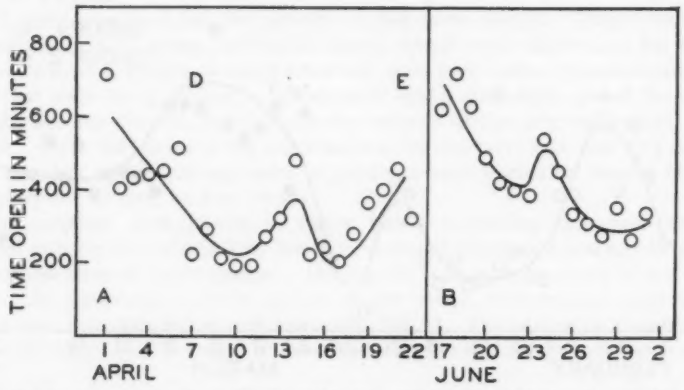


Figure 2. The long-term cycle of activity for group 3 (A) which was studied in Evanston, Ill., and that for group 4 (B) which was studied in Woods Hole, Mass. For explanation of D and E, see text.

2A) and for group 4 (Fig. 2B) show the minor peaks of a cycle with periods of lower activity on either side. It is also evident that the curve for group 3 can be superimposed on those for groups 1 and 2 from points A to C in Figure 1. Furthermore, the curve for group 4 simulates those for groups 1 and 2 from points B to C in Figure 1, and also that for group 3 from D to E in Figure 2A.

It was thought that the clams in each of the first three groups were probably in synchrony with one another since they were reported to have been collected in the same area of the Virginia coast. Examination of the activity records of the individual animals of a single group supports this assumption. Within a group, all the individuals have their maxima and minima occurring within a particular 48-hour period. In group 4, the variation is even less. With the exception of one animal, all the latter showed their highest observed activity on June 17 and a secondary peak of activity on June 24. All the clams of this group had their lowest observed activity

from about June 27 to June 29, correlated with the time of the semi-lunar low, low tides in Little Harbor.

B. The diurnal rhythms

The diurnal rhythms for the four groups of animals are shown in Figure 3. The average percentage of an hour open for each of the 24-hour periods was plotted against the time of day in Eastern Daylight Time, so that the cycles of the Evanston and Woods Hole groups could be compared directly.

Most evident in the curves of the diurnal rhythms are the facts that in all the groups, the maximal activity occurred in the afternoon, and that activity reached a

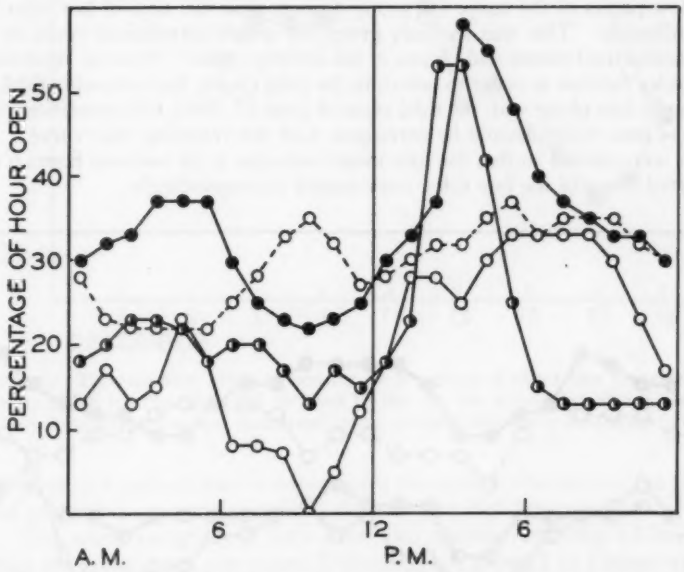


FIGURE 3. The diurnal rhythms of activity for the four groups of animals that were studied under constant conditions. Group 1 (open circles with solid lines), group 2 (black circles with solid lines), group 3 (black and open circles with solid lines), and group 4 (open circles with broken lines).

low point during the morning hours. The afternoon maximum was recorded between 5 and 9 P.M., 3 and 4 P.M., 2 and 4 P.M., and 5 and 6 P.M., for groups 1, 2, 3, and 4, respectively. In groups 1, 2, and 3, the morning minimum occurred between 9 and 10 A.M. whereas in group 4, the time of minimal activity extended from 2 to 6 A.M.

The diurnal rhythm of group 3 is striking in that a relatively long period of low activity is apparent between 7 P.M. and midnight whereas in groups 1, 2, and 4 the activity fell gradually from the afternoon highs during a comparable period of time. All groups of clams also had a minor peak of activity during the morning hours. In

the first three groups these peaks were between 4 and 5 A.M., 3 and 6 A.M., and 2 and 4 A.M., respectively. The morning maximum in group 4 did not occur until 8 A.M. although the morning low occurred earlier, *i.e.*, between 2 and 6 A.M., than in the other groups. The minor peak of the morning occurred about 12 hours before the diurnal maximum in all the groups, except group 4.

C. The tidal rhythms

The tidal rhythms of activity, analyzed as described earlier, are illustrated in Figure 4. Two periods of lower activity are evident in each of these curves. These two periods of each curve are about 12 hours apart, thereby approximately reflecting the normal frequencies of the tides of the Atlantic coast. Also to be noted is the fact that the low points of the curve for group 4 occur near the time of low tides in the area of collection. This was the only group for which correlations could be made between actual tidal events and phases of the activity cycles. Since in rendering the diurnal cycles random in order to ascertain the tidal cycles, the successive tidal cycles were brought into phase with the tidal cycle of June 17, 1953, tidal conditions on this day are the ones which should be correlated with the resulting tidal curve. When the points were moved so that the first minimum came to lie between hours 6 and 7, the indicated times of the low tides were moved correspondingly.

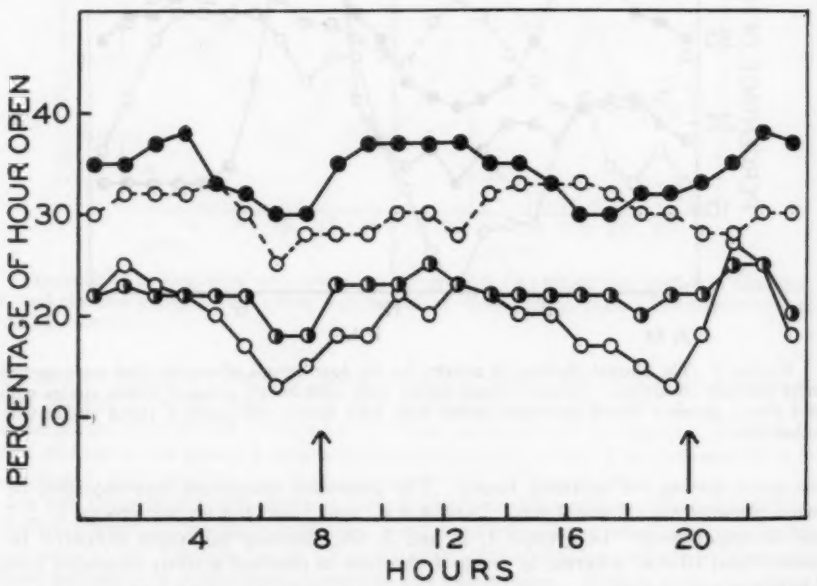


FIGURE 4. The tidal rhythms of activity for the four groups of animals that were studied under constant conditions. Group 1 (open circles with solid lines), group 2 (black circles with solid lines), group 3 (black and open circles with solid lines), and group 4 (open circles with broken lines). Note that the two periods of low activity for group 4 were near the times of low tide as indicated by the arrows.

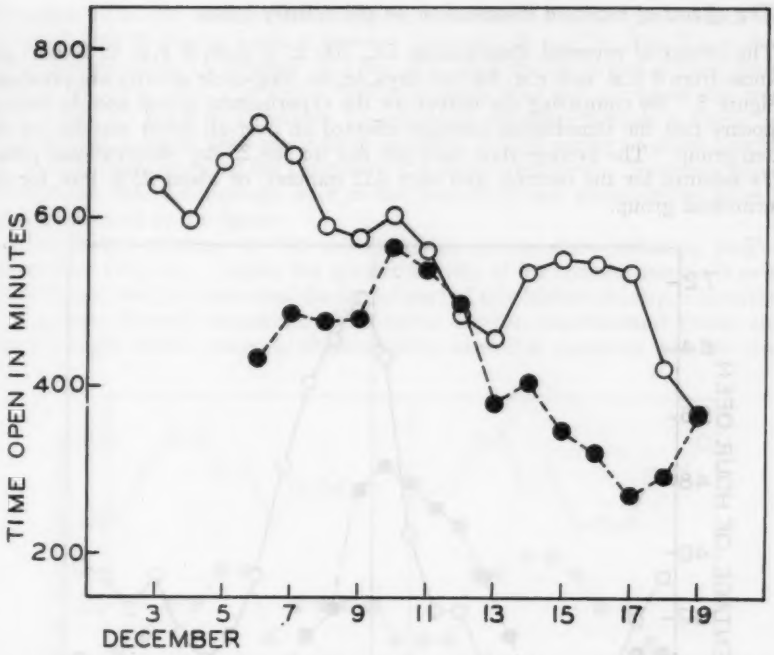


FIGURE 5. The long-term cycles of activity for the group of clams that was exposed to an illumination of 100 ft. c. by night and darkness by day for five consecutive days (broken lines) and for its control group that was maintained under constant laboratory conditions (solid lines).

The particular method used in determining the general character of the tidal cycle does not permit demonstrating precisely the expected 12.4-hour character of the tidal cycles. The curve for group 1 does show two minima occurring 13 hours apart while that for group 2 has low points 10 hours apart, group 3 11.5 hours apart, and group 4 14.5 hours apart. The average obtained, therefore, was 12.25 hours for the four series.

The amplitude of the four cycles also differs from one to another. That for group 1 has the greatest amplitude, an increase of 108% from lows to highs, while the other three increased only 27% to 32% from low points to high ones. Another interesting point evident in the curves of the tidal cycles is that in all of them one of the two periods of increased activity shows two peaks of relatively high activity separated by two to three hours of relatively low activity.

The shapes of the tidal curves for groups 1, 2, and 3 are similar to that illustrated for group 4 for which correlations with actual tidal phases could be made. Therefore, it would be reasonable to predict tidal events in the habitats of groups 1, 2, and 3, since the phases of the persistent tidal cycle probably always bear corresponding relationships to the times of tides in the native habitat.

II. The effects of reversed illumination on the activity cycles

The effects of reversed illumination, *i.e.*, 100 ft. c. from 8 P.M. to 8 A.M. and darkness from 8 A.M. to 8 P.M. for five days, on the long-cycle activity are presented in Figure 5. By comparing the curves for the experimental group and its control, it appears that the illumination changes effected an over-all lower activity for the treated group. The average time open per day for the 20-day observational period is 574 minutes for the controls and only 432 minutes, or about 25% less, for the experimental group.

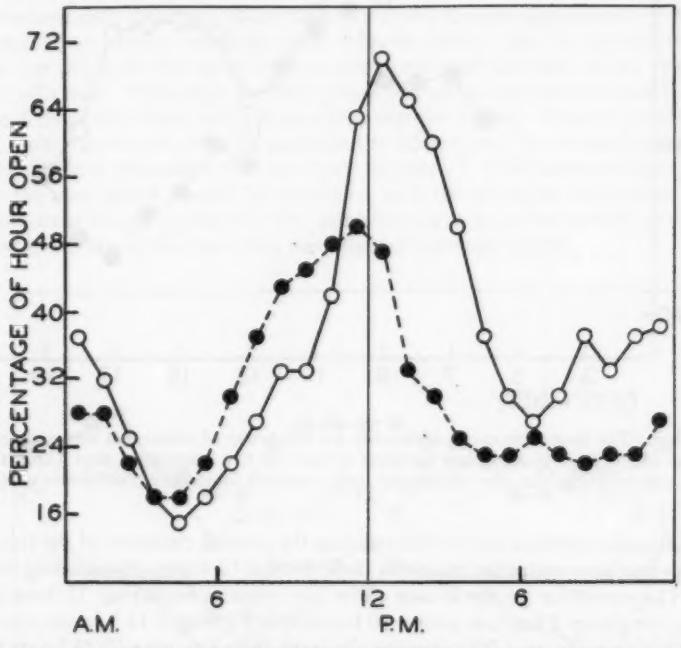


FIGURE 6. The diurnal cycles of activity for the group of clams that was exposed to reversed illumination (broken lines) and for its control (solid lines). The cycle for the experimental group appears to have been shifted one hour to the left or earlier when compared to that for the control group.

A change in the temporal relationships of the curve for the experimental group is also evident in Figure 5. This shift can be seen best by comparing the high point of December 6 on the control curve with a comparable high on December 10 on the experimental one. Low points also occurred four days apart, *i.e.*, on December 13 for the controls and on December 17 for the treated animals.

The general form of the long-term cycle of the control group, as well as that of the experimental group, was seen to be similar to the general form of the long-term cycles of activity that were found for the first four groups of quahogs (Figs. 1 and 2). It is probable that the portion of the cycle from December 13 through

December 19 of the control curve (Fig. 5) represents a minor peak which corresponds to that of groups 1 and 2 between February 18 and 24 (Fig. 1), to that of group 3 from April 11 through 17 (Fig. 2A), and that of group 4 from June 22 through 28 (Fig. 2B). It seems highly probable that the points on the control curve (Fig. 5) prior to December 13 represent the descent from a major peak. If this is true, a major peak would have occurred 15 days earlier or about December 1. A high value recorded for December 1 supports this view. However, this was the first day that the quahogs were in the laboratory and therefore the value was not incorporated in the figure.

The diurnal rhythms for the two December groups were strikingly similar to one another (Fig. 6). Again, the greater activity of the control animals is evident in this figure, and it is seen that the larger part of this higher activity is contributed by a greater diurnal maximum. The curve for the experimental group shows merely a slight shift in temporal characteristics when it is compared with the control

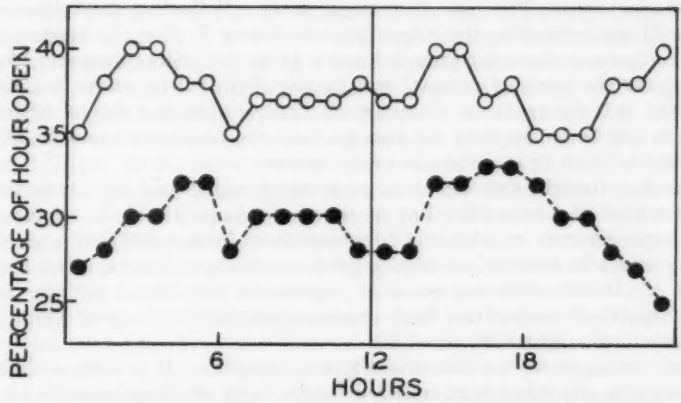


FIGURE 7. The tidal cycles of activity for the group of animals exposed to reversed illumination (broken line) and for the group of its controls (solid lines).

cycle. The diurnal maximum for the experimental group occurred one hour earlier than that for the controls, and the diurnal minimum lasted for two hours (3 to 5 A.M.) in this former group as opposed to one hour (4 to 5 A.M.) in the latter, similarly indicating a small shift to an earlier time.

A comparison of the 24-hour cycles of the two December groups with those of the four groups that were studied earlier in the year (Fig. 3) shows that there are certain likenesses in form, although the temporal aspects of the curves differ. The most obvious difference is that the diurnal maximum for the December groups occurred just before and just after noon whereas the other curves had maxima later in the afternoon. The morning minimum for the December groups was also earlier, *i.e.*, between 3 and 5 A.M., as were the minor peaks of the morning which occurred immediately after midnight. The period between the minor and major peaks, however, was again 12 hours as in groups 1, 2, and 3.

The tidal cycles of the December groups (Fig. 7) are also similar to those of

the earlier groups (Fig. 4). The two minima are separated by about 12 hours and one of the prolonged periods of increased activity is characterized by one or two hours of relatively low activity between periods of higher activity.

It is clearly apparent that the tidal cycle for the group of clams that was exposed to five periods of reversed illumination was shifted in time relative to that for the control group (Fig. 7). Each of the two minima of the experimental curve occurred about five hours later than the comparable lows of the control group. Again, in this figure, the lower average activity of the experimental animals is evident.

Discussion

The evidence at hand supports the assumption that the long-cycle rhythms illustrated in Figures 1, 2, and 5 are actually lunar cycles or parts thereof. The curve drawn between points contributed by groups 1 and 2 shows almost exact lunar periodicity; the maxima are 29 days apart and the minor peak occurs halfway between the two major ones. The time to minimal activity following the minor peak of February 21 and preceding the minor peak of March 7 (Fig. 1) appears shorter than the comparable times for groups 3 and 4 (Fig. 2). Since, however, the overall activity for the clams of group 2 was greater than that for those in group 1, it is suggested that the point for February 25 really constitutes that of minimal activity. On this basis, the time for passage from the lesser maximum to the minimum would be three to four days in every instance.

It was first thought that the clams of group 3 might have been collected from the same region of Chesapeake Bay as those of groups 1 and 2. If these three groups of animals were in tidal, and consequently in lunar synchrony a major peak of activity would be expected to have occurred about April 5 and a minor one about April 20. It is seen from inspection of Figure 2A that this is not the case, but rather an apparent decline from high activity characterizes the cycle about April 5 and a minor peak appears on April 14.

Several explanations for this diversity are plausible. It is very probable that the animals of group 3 had been collected from an area of Chesapeake Bay in which the times of tides differ from those in the region from which the clams of groups 1 and 2 had been obtained. The times of tides in different parts of Chesapeake Bay vary quite considerably because of the topography of this area. Secondly, it is possible that all these animals were collected in the same region but that the rhythms for group 3 were put out of phase by light, temperature, or some other stimulus to which the clams were subjected during shipment from the Atlantic coast to Evanston.

No correlations between the phases of the tidal cycle for group 3 and actual tidal events could be made. Therefore, it is impossible to know whether or not the phases of this tidal rhythm were shifted. Also, the diurnal cycle of activity for group 3 did not differ basically from those for groups 1 and 2. Unpublished observations suggest that for fiddler crabs, the persistent diurnal and tidal rhythms, the summation of which determines the phases of the long-cycle rhythms, are shifted together by temperatures that approach freezing. This is also true of the cycles for fiddler crabs that are shifted by exposure to an illumination of high intensity (Brown, Fingerman, Sandeen and Webb, 1953). Nevertheless, the data on the effects of changes in illumination on the rhythms for quahogs, reported in the present

paper, appear to indicate that the diurnal and tidal cycles were affected differentially by this treatment.

It was seen that the period of minimal activity for group 4 was correlated roughly with the semi-lunar low, low tides which occurred just after the full moon of June 26. It was therefore thought that the long-cycle rhythms for the other groups also might be correlated directly with phases of the moon. This does not seem to be the case, however, since a full moon occurred on February 28, 1953, or about seven days after a minor peak for group 1, on March 28, 1953, or about two weeks before a minor peak for group 3, on June 26, or two days after a minor peak for group 4, and on December 20, or about four days after a minor peak for the group of clams that served as controls in the experiment on effects of reversed illumination. These facts suggest that it is the time of the local tides which determines the phase relationships of the semi-lunar and lunar cycles rather than a more direct lunar influence. This hypothesis is supported by results of the study of the tidal rhythms of color change for *Uca* (Brown, Fingerman, Sandeen and Webb, 1953), which demonstrated that the cycles of crabs that inhabit areas, the tidal times of which differed by about four hours although located in the same geographical region, were correspondingly four hours out of phase with one another. Therefore, the phases of the long-term cycles for quahogs that live in areas of Chesapeake Bay which differ in times of tides would likewise differ from one another, although all the animals would be exposed to the same phases of the moon at the same times.

The similarity of the curve for the tidal cycle for group 4 to those for groups 1, 2, 3, and the group that served as controls in the illumination experiment, and the correlation of the phases of the rhythm of group 4 with actual tidal events, suggest strongly that this same correlation could be made for the latter groups if the exact data on tides were available. All the groups showed diurnal cycles of activity. The investigation by Brown, Fingerman, Sandeen and Webb (1953) proved conclusively that the presence of both persistent tidal and diurnal rhythms in the same animal produces a persistent semi-lunar rhythm since the tidal cycle is in the same relationship with the diurnal cycle at 14.8-day intervals.

If the effect of alternate tidal cycles on metabolism or activity differs quantitatively or qualitatively from that of the intervening tidal cycles, the tidal cycles would be in a similar relationship with the diurnal cycle only at 29.5-day intervals, or lunar ones. This situation appears to be true for the long-term rhythm of the quahogs, since the alternate semi-lunar maxima are larger than the intervening ones. The activity data for only 15 consecutive days were used in the analysis of each of the tidal rhythms. Therefore, when the data for the first 14 days were repeated in the analysis, values which included the effects of both of the daily tides were averaged, and any difference between them was obscured. The tidal rhythm of O_2-consumption for the snail, *Littorina littorea* (Sandeen *et al.*, 1954), was analyzed in the same manner as those reported in the present paper, and it appears to show more clearly than those for the groups of quahogs that the effects of the alternate tidal cycles are not equivalent.

At present, the diurnal cycle of activity of the quahogs cannot be characterized precisely. As has been seen, the activity generally reached a maximum during the afternoon hours and minimal activity occurred during the morning hours. It is possible that the differences that were seen among the diurnal rhythms for the various groups reflect adaptations of the diurnal activity to the varying lengths of

daylight throughout the year. The cycles for groups that were observed during March, April, June, and December show indications that during periods of shorter day-length the greater activity of the clams occurred earlier in the day than it did during the times of the year when days are longer. Activities for groups 2 and 3 which were observed in March and April declined after 4 P.M. Group 4, which was studied in June, showed an hour of maximal activity between 5 and 6 P.M. Correspondingly, the diurnal cycle for the controls for the animals that were exposed to light and dark and were observed in December reached a maximum just after 12 noon. The activity decreased rather rapidly after this time. If this assumption is correct, it is seen that the rhythm for group 1 is not in line with those of the other groups. Group 1 was observed in February at which time daylight hours are fewer than in March and April in the northern hemisphere, but its period of maximal activity extended from 5 P.M. until 9 P.M.

The periods of minimal activity for the different groups of animals also show some correlation with the day-length that existed at the times of observation. For groups 1, 2, and 3, which were observed during the early spring, the diurnal lows were recorded between 9 and 10 A.M., at which times the groups were active 0%, 22%, and 13% of the hour. The period of low activity for group 4, studied in June, ended shortly after 6 A.M., and at this time these animals were active 25% of each hour period. The cycle for the December control group does not fit into this series. Here the morning minimum was reached between 4 and 5 A.M. when the clams were open 16% of the hour. However, these varying diurnal rhythms may be indications of an annual cycle, and yet not reflect day-length directly.

The data for the persistent diurnal rhythms of O_2-consumption for marine snails (Sandeen *et al.*, 1954) and for fiddler crabs (Brown, Bennett and Webb, 1954) suggest that the diurnal patterns may change slightly in accordance with day-length. In these cases, as in the present report, the animals were maintained under constant laboratory conditions. Brown and Stephens (1951) have shown that the day-phase of the diurnal cycle of color change for *Uca* can be influenced by the length of the daily photoperiod to which series of crabs are exposed. The changes in this cycle, once induced, persisted after the animals were placed in constant darkness.

It is interesting to note, once more, that an earlier study of *Venus mercenaria* in its natural environment did not yield any positive suggestions of rhythmic activity (Loosanoff, 1939). It is possible that in an organism's natural habitat, characteristics of the basic persistent rhythms, *e.g.*, frequency, form, and amplitude, are masked or overshadowed by varying external stimuli such as light, temperature, and movements of the water. A suggestion that this may be true is found in the tremendous increase in the amplitude of the diurnal rhythm of color change in *Uca pugnax* after the crabs have been brought from the field and maintained in constant darkness (see Figure 10, Brown, Fingerman, Sandeen and Webb, 1953).

As is true for the persistent rhythms for fiddler crabs, those for *Venus* can be modified to some degree by exposing the animals to alternating periods of darkness and illumination. It has been shown that five consecutive days (8 A.M. to 8 P.M.) of darkness with five nights (8 P.M. to 8 A.M.) of illumination of 100 ft. c. effected changes in the diurnal, tidal, and long-cycle rhythms of activity for a group of clams. Whether or not the apparent shift of the diurnal cycle is significant is difficult to state. As discussed previously, the diurnal rhythms for the different groups of animals that were observed varied to the extent that a precise characterization of this

rhythm could not be made. Furthermore, it is not known whether these variations reflected adaptations to day-length. Therefore, the diurnal cycles of activity illustrated for the experimental group and its control (Fig. 6) may be in phase with one another. However, the moving of both the morning minimum and the afternoon maximum for the experimental group by about the same amount of time and in the same direction, *i.e.*, one hour earlier than the comparable points for the control group, seems to indicate that the phases of this cycle were shifted.

The effects of five consecutive days of darkness and five nights of illumination on the tidal and long-term cycles of activity can be discerned more readily. The tidal rhythm for the experimental group seems to be shifted so that the low points occurred five hours later than the comparable points for the control group (Fig. 7). In other words, the tidal cycle of the experimental animals is about five days ahead of the control one. The phases of the long-term cycle for the experimental clams occurred four days after those of the controls (Fig. 5). Since the long-cycle rhythm is the result of the moving of the tidal influence across the diurnal cycle at the rate of about 50 minutes per day, or so that the tidal effect occurs 50 minutes later on each successively later day, the apparent five-hour shift of the tidal rhythm could not have effected a shift of the long-cycle rhythm such that its phases occurred four days late. If the experimental tidal rhythm had been shifted so that on a hypothetical day the low point of the cycle, which probably correlates with the time of low tide in the natural habitat of the clams, occurred between 11 P.M. and midnight and the comparable low point for the control group occurred five hours earlier, or between 6 and 7 P.M. on the same day, the resulting long-term cycle for the experimental animals would be moved so that its phases would occur earlier rather than later than those of the cycle for the control group.

In order that the phases of the long-cycle rhythm for the experimental group be four days late when compared to those of the control group, the tidal cycle for the former would have had to be shifted so that a low point occurred about four to five hours earlier than, or that the tidal cycle of the experimental group was four to five days behind, that of the control group. As has been seen, this was not the case (Fig. 7). However, when a particular point of the experimental cycle which is the result of a tidal influence appears to be five hours late, or about four to five days ahead, it may in reality be nearly 20 hours early, since a particular tide recurs about every 24.8 hours. If this situation exists, the phases of the long-cycle rhythm for the experimental animals would be expected to occur about 25 days before those of the control cycle (Fig. 8). The facts that a particular tide occurs about 50 minutes later on each successively later day, and that the long-cycle rhythm is repeated about every 29 days, account for this relationship.

Several hypotheses may be offered to explain these shifts. 1) The first dark-to-light or the first light-to-dark change may have been the stimulus that effected the movement of the phases of the tidal cycle for the experimental group about 20 hours backwards, and consequently, the phases of the long-cycle rhythm 25 days ahead of those for the control clams. 2) Possibly, the effects of the changes of illumination or the periods of high illumination or darkness were cumulative, and thereby caused the shifts that have been discussed. On the basis of the results of previous investigations it is probable that the changes from darkness to light and the light period were the effective factors. Webb (1950) found that the phases of the diurnal rhythm of color change for fiddler crabs that were maintained in con-

stant darkness were shifted by single illumination periods of varying lengths. The direction and the amount of shift were dependent on the time of day at which the animals were exposed to an illumination of 90 ft. c. A more recent paper (Brown, Fingerman and Hines, 1954) presents an hypothesis to account for the graded series of shifts of the diurnal cycle of color change for *Uca*. These graded shifts in the phases of the cycle were noted when series of crabs were exposed to various higher illuminations by night and various lower ones by day. Two factors appeared to operate in the altering of the phases: 1) the strength of the light-increase stimulus,

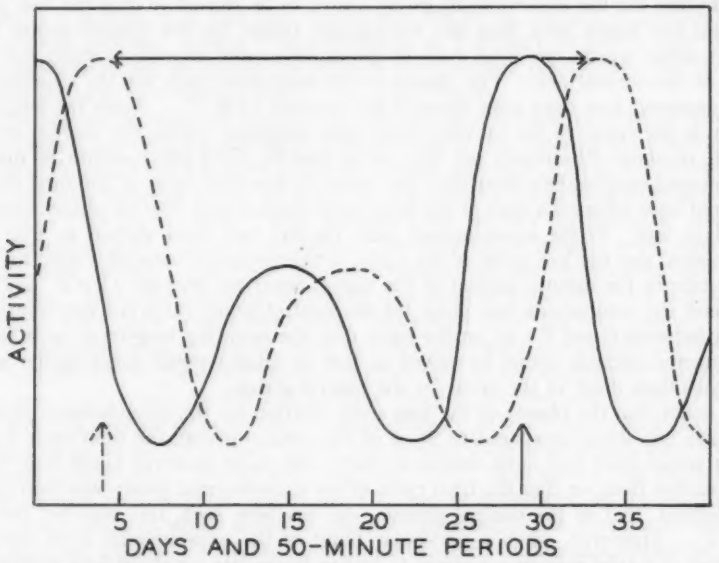


FIGURE 8. An illustration to show the effects of shifts of the tidal cycle of an experimental group of clams on the long-term rhythm of activity. The broken line curve is that of the experimentals and the solid line one is that of the controls. The broken line arrow indicates the time of low tide for the experimental group and the solid line one for the control group. The horizontal arrows indicate the directions and amounts of shifts possible. (For discussion, see text.)

and 2) the brightness of the higher illumination to which the animals were exposed by night.

Exposures of fiddler crabs to several cycles of altered illumination operate to shift the tidal rhythms of color change as well as the diurnal cycles (Brown, Fingerman, Sandeen and Webb, 1953). Here the phases of the tidal and the diurnal rhythms were moved in the same direction and by the same number of hours. In this case, the temporal relationship which existed between the two cycles at the times of exposure of the crabs was maintained. In the present study, the data lead to the assumption that the tidal rhythm for the experimental group of quahogs was shifted so that its phases occurred about 20 hours earlier than those for the animals

that were exposed to constant low illumination. The shift of the diurnal rhythm was, at most, only one hour in the same direction. The diurnal cycle could have been shifted by a number of days, however, and this could not have been ascertained. For example, if each of the five alternating light cycles had shifted the diurnal rhythm backwards by five hours, it would appear to have been set backwards only one hour. Meanwhile, if the tidal influence had each day moved forward 50 minutes over the diurnal cycle while each day the diurnal cycle was set backwards five hours, the phases of the tidal cycle would be in effect set backwards about 20 hours during the five days. This interpretation is so clearly compatible with the second hypothesis advanced earlier that its likelihood is great.

From the results of the present and earlier investigations, it is evident that light stimuli are effective in setting phases of both persistent diurnal and tidal rhythms, and consequently the semi-lunar and lunar ones. Two other investigators recently have discussed the problem of setting the phases of persistent cycles. Brett (1954) saw that a light flash of only one minute was sufficient to set the phases of the diurnal rhythm of emergence for *Drosophila*. The tidal movements of the water seem to participate in determining the phases of the tidal rhythm for *Mytilus* (Rao, 1954).

The question remains: are these persistent rhythms endogenous or exogenous? If they are endogenous, or dependent upon basic physiological mechanisms, the various experimental procedures which shift the phases of the cycles have actually altered the "internal clocks." On the other hand, if the rhythms are controlled by exogenous forces, the manifestations of the rhythms have merely been moved from their normal relationships with the basic cycles when shifts are apparent. The present study contributes no evidence concerning this problem, but studies planned for the near future should provide an answer.

Precise rhythms of activity such as those discussed in the present paper are seemingly adaptive for this species of clam. Loosanoff (1937) states that the gonads of adult *Venus mercenaria* inhabiting Long Island Sound appear to contain ripe sperm or ova throughout the greater part of the year. Yet, spawning does not occur in the majority of animals until August. A certain critical water temperature has been thought to be the stimulus for spawning (Loosanoff, 1937). Although temperature may be a limiting factor it seems more probable that the presence of persistent lunar rhythms, resulting from persisting diurnal and tidal ones in these clams, may eventually be shown to be the immediate regulator of spawning activities. Such a mechanism would provide more precision than could be obtained by the very slow, gradual temperature change of the water.

The greater activity of the clams during the day than during the night may be adaptively correlated with the availability of food. Plankton populations, which supply much of the food of this species, are known to undergo diurnal rhythms of migration. In general, the greatest density of plankters near the surface is found during the hours of the night, and conversely, the greatest density at greater depths occurs during daylight (Russell, 1927).

Summary

1. Continuous kymograph recordings of the opening and closing of the valves of common quahogs, *Venus mercenaria*, secured from the Virginia coast and from

Woods Hole, Massachusetts were made during February, March, April, June, and December, 1953.

2. The activity records of these clams maintained under constant laboratory conditions show a persisting diurnal rhythm with maxima and minima occurring in the afternoon and early morning, respectively. There is some evidence that the pattern of the diurnal cycle varies with the time of year.

3. A persisting tidal rhythm is also evident. In the group of animals for which correlations with actual tidal phases could be made, it was seen that the times of minimal activity in this cycle correspond closely with the times of low tide in the area from which the animals were collected.

4. The summation of these two cycles, diurnal and tidal, is responsible for an observed lunar cycle. The phases of the lunar cycle are probably set directly by the tides to which the animals are exposed rather than by any direct lunar influence.

5. The rhythms of a group of clams that was exposed to darkness during the day (8 A.M. to 8 P.M.) and to an illumination of 100 ft. c. during the night (8 P.M. to 8 A.M.) for five consecutive 24-hour periods show shifts in their cycles when compared to those of control animals. The shifts are such that the phases of the diurnal rhythm occurred one hour earlier than those of the controls; the phases of the tidal cycle occurred about 20 hours earlier than those of the controls; and the phases of the long-cycle rhythm were, in consequence, 25 days ahead of those of the controls.

6. The manner in which these shifts of the phases of the cycles are related is discussed and illustrated.

7. Adaptive significances of these persistent rhythms in these clams are suggested.

LITERATURE CITED

Bohn, G., 1904. Periodicité vitale des animaux soumis aux oscillations du niveau des hautes mers. *C. R. Acad. Sci., Paris,* **139**: 610–611.

Brett, W. J., 1954. Persistent diurnal rhythmicity in *Drosophila* emergence. *Ann. Ent. Soc. Amer.,* in press.

Brown, F. A., Jr., 1954. A simple, automatic, continuous-recording respirometer. *Rev. Sci. Instruments,* **25**: 415–417.

Brown, F. A., Jr., H. M. Webb, M. F. Bennett and M. I. Sandeen, 1954. Temperature independence of the frequency of the endogenous tidal rhythm of *Uca*. *Physiol. Zool.,* in press.

Brown, F. A., Jr., M. F. Bennett and H. M. Webb, 1954. Persistent daily and tidal rhythms of O_2-consumption in fiddler crabs. *Jour. Cell. Comp. Physiol.,* in press.

Brown, F. A., Jr., M. Fingerman and M. N. Hines, 1954. A study of the mechanism involved in shifting of the phases of the endogenous daily rhythms by light stimuli. *Biol. Bull.,* **106**: 308–317.

Brown, F. A., Jr., M. Fingerman, M. I. Sandeen and H. M. Webb, 1953. Persistent diurnal and tidal rhythms of color change in the fiddler crab, *Uca pugnax*. *J. Exp. Zool.,* **123**: 29–60.

Brown, F. A., Jr., and M. N. Hines, 1952. Modifications in the diurnal pigmentary rhythm of *Uca* effected by continuous illumination. *Physiol. Zool.,* **25**: 56–70.

Brown, F. A., Jr., and G. C. Stephens, 1951. Studies of the daily rhythmicity of the fiddler crab, *Uca*. Modification by photo-period. *Biol. Bull.,* **101**: 71–83.

Brown, F. A., Jr., and H. M. Webb, 1948. Temperature relations of an endogenous daily rhythmicity in the fiddler crab, *Uca*. *Physiol. Zool.,* **21**: 371–381.

Brown, F. A., Jr., and H. M. Webb, 1949. Studies of the daily rhythmicity of the fiddler crab, *Uca*. Modifications by light. *Physiol. Zool.,* **22**: 136–148.

GOMPEL, M., 1937. Recherches sur la consommation d'oxygène de quelques animaux aquatiques littoraux. *C. R. Acad. Sci., Paris,* **205**: 816–818.

KLEITMAN, N., 1949. Biological rhythms and cycles. *Physiol. Rev.,* **29**: 1–30.

KORRINGA, P., 1947. Relations between the moon and periodicity in the breeding of marine animals. *Ecol. Monogr.,* **17**: 349–381.

KORRINGA, P., 1952. Recent advances in oyster biology. *Quart. Rev. Biol.,* **27**: 266–308.

LOOSANOFF, V. L., 1937. Seasonal gonadal changes of adult clams, *Venus mercenaria* (L.). *Biol. Bull.,* **72**: 406–416.

LOOSANOFF, V. L., 1939. Effect of temperature upon shell movements of clams, *Venus mercenaria* (L.). *Biol. Bull.,* **76**: 171–182.

PARK, O., 1940. Nocturnalism—The development of a problem. *Ecol. Monogr.,* **10**: 485–536.

RAO, K. P., 1954. Tidal rhythmicity of rate of water propulsion in *Mytilus,* and its modifiability by transplantation. *Biol. Bull.,* **106**: 353–359.

RUSSELL, F. S., 1927. The vertical distribution of plankton in the sea. *Biol. Rev.,* **2**: 213–262.

SANDEEN, M. I., G. C. STEPHENS AND F. A. BROWN, JR., 1954. Persistent daily and tidal rhythms of O_2-consumption in two species of marine snails. *Physiol. Zool.,* in press.

STEPHENS, G. C., M. I. SANDEEN AND H. M. WEBB, 1953. A persistent tidal rhythm of activity in the mud snail, *Nassa obsoleta. Anat. Rec.,* **117**: 635.

WEBB, H. M., 1950. Diurnal variations of response to light in the fiddler crab, *Uca. Physiol. Zool.,* **23**: 316–337.

WELSH, J. H., 1938. Diurnal rhythms. *Quart. Rev. Biol.,* **13**: 123–139.

ON THE RESPIRATION IN SCALLOPS (LAMELLIBRANCHIATA)[1]

L. VAN DAM

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

The only data in the literature on the respiration of scallops seem to be those of Spärck (1936; *cf.* also Thorson, 1936), who determined the oxygen uptake in *Pecten groenlandicus* (arctic), *P. varius* (boreal-mediterranean), and *P. flexuosus* (mediterranean) in nearly air-saturated water. This author found that the resting metabolism of the scallops, and other bivalves which have a marked capacity for rapid swimming locomotion, is considerably higher than that of more sedentary types.

The present paper reports on a study of the respiration in the deep sea scallop and the bay scallop, both of common occurrence and of considerable economic importance along certain areas of the Atlantic coast of North America. The oxygen consumption of resting (non-swimming) individuals was determined over a range of concentrations of oxygen of about the air-saturation value to close to zero. In addition determinations were made, in aerated water, of the oxygen utilization or the percentage of oxygen removed from the inspired water during its passage through mantle cavity and gills.

Material and Method of Oxygen Analysis

The deep sea scallops (*Pecten grandis* Solander) were taken from Cape Cod Bay, at a depth of about 12 m., the bay scallops (*Pecten irradians* Lamarck) from a shallow salt water pond near Woods Hole. All animals were kept in the laboratory in tanks with running sea water (salinity 31–32.5‰). For the experiments, which were carried out during June and July, 1952, and from September–November, 1953, only specimens that were in apparent good health, showing a wealth of actively moving, extended tentacles and production of faecal material, were used.

Analyses of dissolved oxygen were made by the Winkler method on samples of about one ml. (van Dam, 1935a).

Techniques of Measurements of O_2-Consumption

(a) Continuous measurements of O_2-consumption in water approximately saturated with air were performed on single individuals of the sea scallop by means of a volumetric respirometer technique as described by Scholander (1949). In this method the oxygen consumed is directly read on a syringe from which oxygen is introduced into the system at the rate at which it is removed by the animal.

A plastic box of about 900 ml. capacity, immersed in a water bath, was used as a respiration chamber. It was filled with about 850 ml. of sea water which had been filtered through cotton or glass wool. Prior to the actual experiments the animals were kept for one hour or longer in the respirometer running open to the

[1] Contribution no. 705 from the Woods Hole Oceanographic Institution.

air. Runs without an animal in the water showed a blank oxygen consumption of, on the average, about 0.2 ml. O_2/hr. (Fig. 5). The cause of these rather high blank values is not known. The calculated Q_{O_2}-values (O_2-consumption per kg. and hour) given in Figure 4 are STP- and blank-corrected. In a number of tests initial and final O_2-content and approximate pH (Hellige comparator) of the water in the respirometer were determined, showing that, as a rule, the O_2-content remained practically constant. In a few cases a decrease of 0.1–0.3 ml. O_2/L. was observed, and the Q_{O_2}-values were corrected accordingly. Although in this method the CO_2 produced is continuously being removed, the pH of the water during the tests usually decreased from about 8 to about 7.2–7.6. This, however, as can be seen from Figure 5, had no apparent effect on the rate of oxygen uptake. The same holds for the experiments with the closed respiratory chamber technique described below, in which all excreta, CO_2 included, remained in the water.

(b) To determine the critical O_2-tension, measurements of O_2-consumption were made in closed plastic respiration chambers of 1–3 L. capacity (Fig. 1), completely

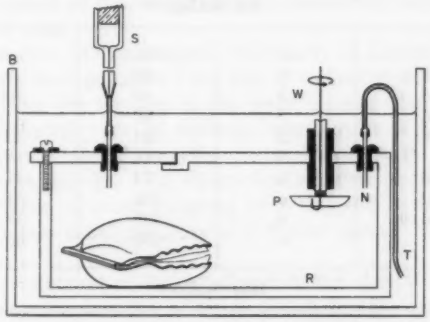


FIGURE 1. Diagram of closed respiratory chamber (R). W, P: stainless steel drive shaft and plastic paddle. T, N: small bore plastic tubing and needle through which water from water-bath B enters R when water samples for O_2-analysis are taken with syringe S.

filled with water, in which the concentration of oxygen decreased progressively due to the respiration of the animal.

Blank runs (Fig. 6) showed that in the absence of an experimental animal the O_2-content of the water in the respirometer was practically constant at any O_2-tension. Control tests showed that the Winkler procedure, as such, was not interfered with by metabolites in water in which animals had been confined for several hours.

Prior to the O_2-uptake measurements the animals were scrubbed clean thoroughly and acclimated to the experimental temperatures for one day or longer. To determine the wet weight of the soft tissues, the animal was opened by severing the adductor muscle. Then all soft tissues were taken out of the shells, drained for about 5 minutes, dried at the outside on filter paper and then weighed. Length and weight of the animals used are given in Table I.

Technique of Determining the Oxygen Utilization

The sea scallops were lying freely in a normal position, *i.e.*, on the right valve, on the bottom of an all-glass aquarium. Samples of the inhalant water current were taken with a syringe, the tip of which was placed near the pallial opening opposite the middle of the hinge. Simultaneously, samples of the exhalant current were taken, either directly by means of a syringe to which a fine glass tip, bent at right angles, was attached (animals no. 1 and 5, 18 samples), or by means of a siphon as

Table I

*Length and weight of experimental animals**

Specimen no.	Number of test in which specimen was used		Length (mm.)	Wet meat (gms.)	Total wet weight (gms.)
	Test no.	Fig. no.			
Sea Scallop					
1	—	—	146	—	—
2	1–4	5	59	9	25.0
3	7, 8	5	63	10.5	29.5
4	5, 6	5	69	13.5	40.0
5	9–13	5	81	23.0	88.0
6	14, 15	5	75	18.0	50.0
7	7, 8	6	73	17.8	49.0
8	1, 2	6	101	34.5	110
9	3, 6, 9	6	139	78.8	294
11	11	6	130	60.3	235
Bay Scallop					
1	—	—	56	6.4	31.9
2	—	—	56	7.0	33.5
3	4, 10	6	61	10.2	38.1
5	5, 13	6	63	12.5	39.0
6	5, 12	6	59	10.5	33.8

* The length given here denotes the straight-line distance between the middle of the hinge (p) and a point at the margin of the upper shell opposite p. Due to circumstances, the wet meat weight of the sea scallops 2, 3, 4, 5 and 6 was not determined directly but was taken from a graphic representation of data, collected by Mr. J. A. Posgay, relating length and weight of wet meat in over one hundred specimens.

indicated in Figure 2 (animal no. 6, 12 samples). Glass tip or siphon were introduced about 5 mm. into the cloacal chamber. Water from the cloacal cavity was allowed to pass through the siphon (capacity 7 ml.) for about one hour, at a rate of 1.5 ml./min., before the first samples were taken. There were no signs that the presence of tip or siphon, which, as a rule, did not touch the animal's tissues, interfered with the normal vigorous propulsion of water. Slowness of sampling guaranteed that the sample was not contaminated with outside water. A continuous flow of fresh sea water combined with an intensive stirring by means of big bubbles

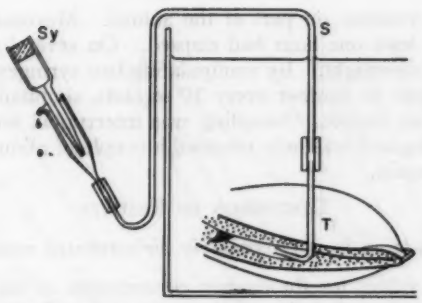


FIGURE 2. Sampling from the exhalant water current in the sea scallop by means of a cloacal siphon. From the continuously overflowing water, samples of about one ml. are taken with a syringe pipette (Sy). The lower end of the glass tube T is about 5 mm. inside the (white) cloacal chamber. Stippled: mantle. Black: tip of gills. Diagram.

of air kept the O_2-content of the water in the experimental aquarium uniform over prolonged periods of time.

In the earlier studies of the oxygen utilization in Lamellibranchia, made on siphonate forms, it has been assumed that, due to turbulent mixing in the relatively long and narrow siphon, the oxygen in the water leaving the exhalant aperture is fairly uniformly distributed. In the scallops, however, as in many other species, a siphon is lacking and the cloacal chamber communicates with the exterior through a normally widely opened slit. To determine whether appreciable O_2-gradients occur in the broad ribbon of water escaping from this slit, in the bay scallop two or three samples were taken simultaneously at different spots. The technique used is indicated in Figure 3.

As a rule the introduction of the needles into the cloacal cavity disturbed the ventilation only during the first few minutes and in several cases was accomplished

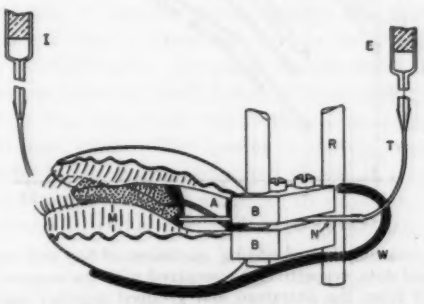


FIGURE 3. Sampling from the exhalant water current in the bay scallop by means of 2 or 3 needles (N), held, in a parallel fashion, 3–10 mm. apart, in a plastic block (B). The needles, of which only one is shown, protrude ½–1½ cm. into the cloacal chamber (black), and are connected with a sampling syringe (E). The scallop is paraffined onto a piece of wire (W), held in a plastic rod (R). I: One of the two syringes, sampling simultaneously, at different spots, from inhalant current. M: mantle. Stippled: gills. A: adductor muscle. Hatched: intestine, opening, at the left hand side, into the cloacal chamber (black). Diagram.

without any visible reaction on part of the animal. Moreover, sampling was not begun until after at least one hour had elapsed. On several occasions the needles were left in position overnight. By manipulating two syringes at a time and changing over from one pair to another every 10 seconds, simultaneity of sampling was accomplished to a fair degree. Sampling was interrupted when an animal would interrupt the pumping and was only resumed a couple of minutes after normal ventilation had begun again.

Discussion of Results

(*a*) *Oxygen consumption in approximately air-saturated water*

In Figure 4 the values for the oxygen consumption of the sea and bay scallop obtained in the present study (dots and crosses, respectively) are compared with data on the oxygen uptake of various other marine Lamellibranchia selected from the literature. The latter were grouped together and averaged according to geographical distribution (arctic, boreal, and mediterranean) and locomotory faculties (swimming versus non-swimming) of the animals. The relation between these

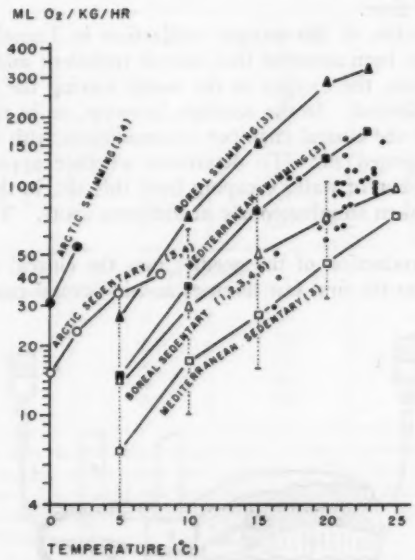


Figure 4. Oxygen consumption of resting specimens of bay and sea scallop studied in the present paper (crosses and dots, respectively) compared with the oxygen uptake of other marine Lamellibranchia, selected from the literature and grouped together according to geographical distribution (arctic, boreal and mediterranean species) and according to locomotory faculties (swimming or more or less sedentary species). On the curves, the points represent the arithmetical mean of all Q_{O_2}-values available for a given temperature. The figures in brackets refer to the source in the literature from which the data, used to compute these mean values, were taken, as follows: 1 = Bruce, 1926; 2 = Thamdrup, 1935; 3 = Spärck, 1936; 4 = Thorson, 1936; 5 = Whedon and Sommer, 1937. Vertical, stippled, lines: spread of data in the group of the boreal sedentary species.

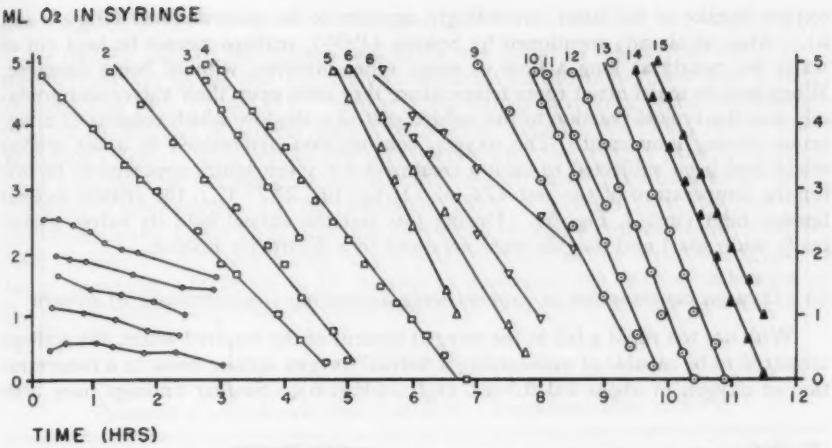


FIGURE 5. Rate of oxygen uptake in the sea scallop in approximately air-saturated water. Ordinate: amount of oxygen (ml.) in the measuring syringe. Abscissa: time (hrs.). Numbering of tests as in Table I. Small circles: blank runs.

factors and the metabolic rate in marine Lamellibranchia has been dealt with already by Spärck (1936). The following data (adult specimens, outside the spawning season) were used: Spärck (1936): 9 arctic, 12 boreal, and 11 mediterranean species; Thorson (1936): 11 arctic species; Thamdrup (1935): 3 boreal species; Bruce (1926): one boreal species; and Whedon and Sommer (1937): one boreal species. The figure indicates that the O_2-consumption in the bay scallop is essentially the same as that in the sea scallop. Over the range of temperatures investigated (16–24° C.), the O_2-uptake in these species is somewhat intermediate in position between the O_2-uptake of mediterranean scallops and that of more sedentary bivalves from that area and thus is about the same as the O_2-uptake of the average boreal non-swimming lamellibranch. In using the curves in Figure 4 as a basis for comparison, it must be borne in mind, however, that they give a greatly simplified picture inasmuch as the data, from which they were computed, show a very considerable spread in most cases. The spread in the Q_{O_2}-values of the different species of lamellibranchs in the boreal-sedentary group, is indicated in the figure (stippled lines). Not enough data are available to establish, in the bay and sea scallop, a relation between Q_{O_2} and season or size, or to estimate Q_{10}.

For several species of bivalve molluscs, lengthy periods of stoppage of the water propulsion have been reported in the literature. To the list of species given by van Dam (1938, p. 123; *cf.* also Verwey 1952, pp. 189–193) can be added *Venus mercenaria* (Collip, 1921; Chipman and Galtsoff, 1949) and *Hyridella australis* (Hiscock, 1950; 1953). Persistence of rhythmicity in O_2-uptake in molluscs under laboratory conditions was further reported by Gompel (1937) and by Sandeen *et al.* (1953), and in the rate of water propulsion in the California mussel by Rao (1953). No prolonged interruptions of the ventilation were observed in the scallops and the

oxygen uptake of the latter, accordingly, appears to be quite uniform (Figs. 5 and 6). Also, as already mentioned by Spärck (1936), scallops cannot be kept out of water for nearly as long a time as many other bivalves, without being damaged. When kept in moist air at room temperature they soon open their valves and gradually lose the typical reaction to the sudden cast of a shadow which consists of a vigorous closing movement. The oxygen consumption determined in a sea scallop which had been subjected to such a treatment for seven hours appeared to be low for the temperature of the test (74 ml. O_2/kg./hr., 22.7° C.), the critical oxygen tension high (test 3, Fig. 6). During this test the animal held its valves abnormally wide apart and its gills were arranged in a disorderly fashion.

(*b*) *Oxygen consumption at progressively decreasing concentrations of oxygen*

With not too rapid a fall in the oxygen content of the inspired water, the scallops appeared to be capable of maintaining a normal oxygen uptake down to a concentration of oxygen of about 1.0–0.5 ml. O_2/L. (Fig. 6). Similar findings have been

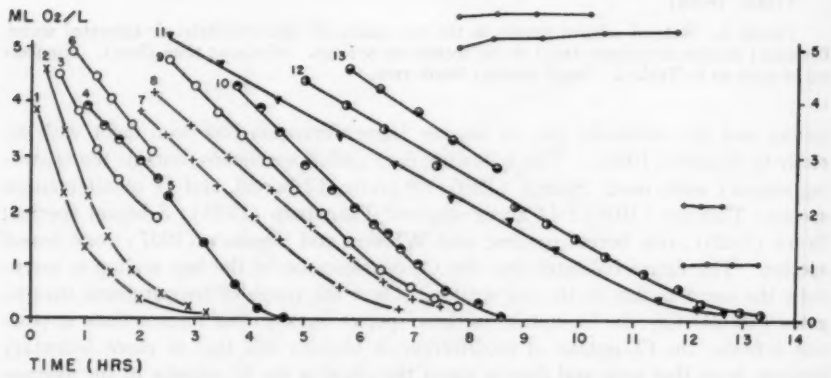


FIGURE 6. Rate of oxygen uptake of the sea scallop and of the bay scallop at progressively decreasing tensions of oxygen. Ordinate: O_2-content of water in respiratory chamber (ml. O_2/L.). Abscissa: time (hrs.). Numbering of tests as in Table I. For discussion of test no. 3, see text. Small circles: blank runs.

reported for other species of Lamellibranchiata (Weiland, 1919; Nozawa, 1929; Galtsoff and Whipple, 1930; Ishida, 1935; Thamdrup, 1935; Whedon and Sommer, 1937; Hers, 1943). To what extent this faculty is based on an increase in the amount of water pumped or on an increase in the percentage oxygen utilization, has not been investigated. It was noticed that the animals, in water poor in oxygen, display little or no active motion, gradually lose the "shadow-cast reaction," and erect the pallial velum in a vertical fashion. It is possible that the vigorous stirring of the water in the respiration chamber has contributed to some extent to the ventilation of the animal's tissues with water and thus has lowered the critical oxygen tension below a level which would prevail if this ventilation depended solely on the currents generated by the animal itself.

(c) *Oxygen utilization*

In the sea scallops the oxygen utilization was found to range from about ½ to 9%; in four out of the five bay scallops investigated the values represent a range of about 1 to 13% (Table II). Similarly low values have been found in siphonate Lamellibranchia and in several other filter feeding animals (van Dam, 1935b, 1938; Hazelhoff, 1938). Low values are also obtained for the oxygen utilization calculated from data on oxygen consumption and rate of water propulsion as given for a number of filter feeders, e.g., by Jørgensen (1952). It seems, then, that true filter feeders normally pump large amounts of water (*cf.* also Loosanoff and Nomejko, 1946; Owen, 1953), from which only a small fraction of the dissolved oxygen is being consumed. It would be of interest to determine the oxygen utilization or the rate of water propulsion in those species of bivalves which are not true filter

TABLE II

*Percentage oxygen utilization in the sea scallop**
O_2-content of inhaled water about 5 ml. O_2/L. Temperature about 17–22° C.

Specimen no. 1	Specimen no. 5	Specimen no. 6
2.9	7.5	1.9
2.2	6.4	2.7
0.7	5.2	0.8
1.6	7.6	3.6
1.6		2.3
1.6	Mean: 6.6%	2.3
4.0		2.9
8.8		3.4
1.8		1.9
4.0		3.8
2.2		3.8
2.2		3.8
0.4		
1.0		Mean: 2.8%
Mean: 2.5%		

* Except for one (Specimen no. 4, utilization 0.9%), the utilization values obtained in the bay scallop are shown in Figure 7.

feeders as, for example, those feeding on bottom deposits (cf. Yonge, 1953).

In one of the bay scallops which ventilated much less vigorously than any of the other specimens investigated, high percentage oxygen utilization values (13–72%) were found. A similar, slow, often interrupted, ventilation entailing a high oxygen utilization was observed in some individuals of Anodonta (van Dam, 1938). In the specimens of Anodonta investigated by Koch and Hers (1943; personal communication) a high utilization prevailed throughout. Whether a slow rate of ventilation, as displayed by these specimens of Pecten and Anodonta, can be considered normal, *i.e.*, will provide the animal with enough oxygen and food to sustain normal, aerobic, respiration and growth, is not known.

In the stream of water escaping from the cloacal chamber of the bay scallops considerable gradients in the concentration of oxygen occur. These gradients are

shown, in Figure 7, in the form of utilization values obtained simultaneously, but at different spots, in the exhalant water current of one and the same specimen. These values were connected by a line parallel to the ordinates. The steepest oxygen concentration gradients occurred in those cases where the utilization was high. In one of these cases (the group of three dots closest to the right hand ordinate) the utilization values were 33.4%, 37.4%, and 71.5%, respectively, representing oxygen concentrations of 66.6%, 62.6%, and 28.5%, of the oxygen content of the inhaled water. In this case, then, the concentration of oxygen at one spot was more than twice as high as at another spot. In the other, more vigorously ventilating specimens, in which the utilization was much lower, the differences in oxygen concentration did not exceed 10% of the O_2-content of the inhaled water.

Inhomogeneity in the distribution of oxygen in the exhalant water current as observed in the bay scallop by the present author and in *Anodonta* by H. J. Koch

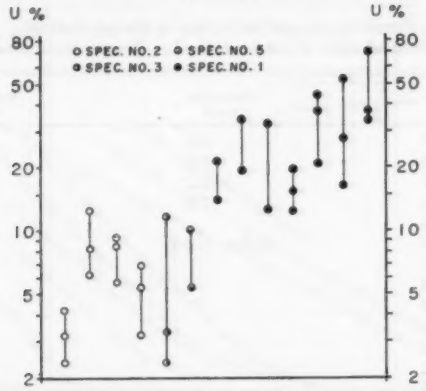


FIGURE 7. Gradients in the concentration of oxygen in the exhalant water current in the bay scallop, presented as the percentage oxygen depletion (U%) of samples drawn simultaneously at different spots in one and the same animal. U%-values from simultaneously drawn samples are connected by a line parallel to the ordinates. O_2-content inhaled water approx. 4.5–5 ml. O_2/L.; temperature about 20–24.5° C.

and M. J. Hers (personal communication), of course, excludes the possibility of determining the true average oxygen utilization with a sampling method. Only if all the water leaving the animal could be collected would the determination of such a value be possible. The oxygen utilization values, then, obtained in this and in previous investigations, are only approximations of this average value.

I wish to thank Dr. A. C. Redfield and Dr. P. F. Scholander for stimulating interest and valuable criticism, and Mr. J. A. Posgay for help in the procurement of scallops.

SUMMARY

1. The oxygen uptake and the percentage of oxygen withdrawn from the inhaled water, were determined in resting specimens of two species of lamellibranch mol-

luscs which have a capacity for rapid swimming locomotion, *viz.* in the sea scallop (*Pecten grandis* Sol.) and in the bay scallop (*Pecten irradians* Lam.).

2. In accordance with the absence of prolonged ventilation pauses common in several other species of Lamellibranchia, the oxygen uptake in the bay and sea scallop is quite uniform. In both species Q_{O_2} was found to be about 70 ml./kg./hr. at 20° C. This value is about half the Q_{O_2}-value found in swimming species from the mediterranean and falls well within the range of Q_{O_2}-values in boreal non-swimming types of lamellibranchs recorded in the literature.

3. The oxygen uptake was independent of the oxygen tension down to a concentration of oxygen of about 1-½ ml. O_2/L.

4. The percentage of oxygen withdrawn from the inhaled water, in most cases, was low, ranging, approximately, from ½ to 13%. It was demonstrated that in the stream of water escaping from the cloacal chamber of the bay scallop considerable gradients in the concentration of oxygen occur.

LITERATURE CITED

Bruce, J. R., 1926. The respiratory exchange of the mussel (*Mytilus edulis* L.). *Bioch. J.*, **20**: 829–846.

Chipman, W. A., and P. S. Galtsoff, 1949. Effects of oil mixed with carbonized sand on aquatic animals. *U. S. Dep. Int. Fish and Wildlife Serv. Spec. Sc. Rep. Fish.*, **1**: 1–52.

Collip, J. B., 1921. A further study of the respiratory processes in *Mya arenaria* and other marine mollusca. *J. Biol. Chem.*, **49**: 297–310.

Galtsoff, P. S., and D. V. Whipple, 1930. Oxygen consumption of normal and green oysters. *Bull. U. S. Bur. Fish.*, **46**: 489–508.

Gompel, M., 1937. Recherches sur la consummation d'oxygène de quelques animaux aquatiques littoraux. *C. R. Acad. Sci.*, **205**: 816–818.

Hazelhoff, E. H., 1938. Ueber die Ausnutzung des Sauerstoffs bei verschiedenen Wassertieren. *Zeitschr. vergl. Physiol.*, **26**: 300–327.

Hers, M. J., 1943. Relation entre respiration et circulation chez *Anodonta cygnea* L. *Ann. Soc. Roy. Zool. Belgique*, **74**: 45–54.

Hiscock, I. D., 1950. Shell movements of the freshwater mussel *Hyridella australis* Lam. (Lamellibranchiata). *Austr. J. Mar. Freshwater Res.*, **1**: 259–268.

Hiscock, I. D., 1953. Osmoregulation in Australian freshwater mussels (Lamellibranchiata). II. Respiration and its relation to osmoregulation in *Hyridella australis* (Lam.). *Austr. J. Mar. Freshwater Res.*, **4**: 330–342.

Ishida, S., 1935. On the oxygen consumption in the oyster *Ostrea gigas* Thunberg under various conditions. *Sci. Rep. Tôhoku Univ., Ser. 4*, **10**: 619–638.

Jørgensen, C. B., 1952. On the relation between water transport and food requirements in some marine filter feeding invertebrates. *Biol. Bull.*, **103**: 356–363.

Koch, H. J., and M. J. Hers, 1943. Influence de facteurs respiratoires sur les interruptions de la ventilation par le siphon exhalant chez *Anodonta cygnea* L. *Ann. Soc. Roy. Zool. Belgique*, **74**: 32–44.

Loosanoff, V. L., and C. A. Nomejko, 1946. Feeding of oysters in relation to tidal stages and to periods of light and darkness. *Biol. Bull.*, **90**: 244–264.

Nozawa, A., 1929. The normal and abnormal respiration in the oyster *Ostrea circumpicta* Pils. *Science Rep. Tôhoku Imp. Univ., Ser. 4*, **4**: 259–325.

Owen, G., 1953. On the biology of *Glossus humanus* (L.) (Isocardia Cor Lam.). *J. Mar. Biol. Assoc.*, **32**: 85–106.

Rao, K. P., 1953. Rate of water propulsion in *Mytilus californianus* as a function of latitude. *Biol. Bull.*, **104**: 171–181.

Sandeen, M. I., G. C. Stephens and F. A. Brown, Jr., 1953. Persistent diurnal rhythms of O_2-consumption in the periwinkle, *Littorina littorea*, and the oyster drill, *Urosalpinx cinereus*. *Biol. Bull.*, **105**: 381–382.

SCHOLANDER, P. F., 1949. Volumetric respirometer for aquatic animals. *Rev. Sci. Instr.*, **20**: 885–887.

SPÄRCK, R., 1936. On the relation between metabolism and temperature in some marine lamellibranchs, and its zoogeographical significance. *K. Danske Vid. Selsk. Biol. Medd.*, **13** (5): 1–27.

THAMDRUP, H. M., 1935. Beiträge zur Ökologie der Wattenfauna auf experimenteller Grundlage. *Medd. Komm. Danmarks Fiskeri og Havunders, Ser. Fiskeri*, **10**: 2.

THORSON, G., 1936. The larval development, growth, and metabolism of arctic marine bottom invertebrates compared with those of other seas. *Medd. om Gronland*, **100**: 1–155.

VAN DAM, L., 1935a. A method for determining the amount of oxygen dissolved in 1 cc. of water. *J. Exp. Biol.*, **12**: 80–85.

VAN DAM, L., 1935b. On the utilization of oxygen by *Mya arenaria*. *J. Exp. Biol.*, **12**: 86–94.

VAN DAM, L., 1938. On the utilization of oxygen and regulation of breathing in some aquatic animals. Thesis, Groningen.

VERWEY, J., 1952. On the ecology of distribution of cockle and mussel in the Dutch Waddensea. Their role in sedimentation and the source of their food supply. With a short review of the feeding behaviour of bivalve molluscs. *Arch. Neérl. Zool.*, **X**: 171–239.

WEINLAND, E., 1919. Beobachtungen ueber den Gaswechsel von *Anodonta cygnea* L. *Zeitschr. Biol.*, **69**: 1–86.

WHEDON, W. F., AND H. SOMMER, 1937. Respiratory exchange of *Mytilus californianus*. *Zeitschr. vergl. Physiol.*, **25**: 523–528.

YONGE, C. M., 1953. Mantle chamber and water circulation in the Tridacnidae (Mollusca). *Proc. Zool. Soc. London*, **123**: 551–561.

STUDIES ON THE ACROSOME. II. ACROSOME REACTION IN STARFISH SPERMATOZOA [1]

JEAN C. DAN

Misaki Marine Biological Station, Misaki, Kanagawa-ken, Japan

With the technical assistance of A. KITAHARA and T. KOHRI

After it had been found that sea urchin spermatozoa react to species egg-water by undergoing a change in the region of the acrosome simultaneously with the agglutination reaction, the possibility became evident that some such reaction might also be occurring in starfish spermatozoa. Fertilization in this form holds a peculiar interest, both because it was in the eggs of the starfish, *Asterias,* that Fol for the first time, in 1877, observed the entrance of a spermatozoan into an egg cell, and because his description of the process, which has never been superseded, contains certain points which are rather difficult to reconcile with usual concepts of the relationship between egg and spermatozoan.

Current ideas concerning the exact details of the process by which fertilization takes place in starfish eggs are in a somewhat surprising state of confusion, considering the number of embryologists who have observed and reported the phenomenon, and the extent to which this material has been used in various studies during the past seventy-five years.

Fol (1877) and Chambers (1923) observed spermatozoa being drawn toward a cone on the egg surface by a long, slender filament, which they believed to be an extension of the cone. Just (1929) repudiated this interpretation, and maintained that the much shorter filament which he observed originates in the sperm head. Hörstadius (1939) reported that a tubular "Empfängnishügel" grows out from the egg surface and takes possession of a spermatozoan at the outer edge of the jelly layer.

Similarly, although workers from the time of Lillie and Loeb have attempted to observe agglutination of starfish sperm in homologous egg-water, their results do not agree. Glaser (1914) and Woodward (1918) reported agglutination in *Asterias forbesii,* and Nomura (1926), in *Asterina pectinifera,* but Just (1930) was unable to confirm the *Asterias* results, and Loeb (1914) and recently Tyler (1941) have also reported lack of success with *Asterias ochraceus* and *Patiria miniata,* respectively. However, in 1944, Metz discovered that the addition of lobster serum caused an unequivocal agglutination reaction in the presence of homologous egg-water. Metz (1945) has further studied the phenomenon in four species of starfish, and found other substances which also act as adjuvants, particularly isotonic white of hen's egg.

The work reported in this paper was planned to test the effect of egg-water (plus an adjuvant) on the acrosome of the starfish spermatozoan.

[1] This research was supported in part by the Ministry of Education Research Expenditure (Min-kan Kenkyu Hi).

Material and Methods

The starfish species used for the majority of the observations were *Asterina pectinifera* and *Asterias amurensis;* the main points were corroborated in *Astropecten scoparius.* Since the oocytes of *Asterina* and *Astropecten* in general do not begin the maturation divisions on suspension in sea water,[2] the egg-water in experiments with these forms was obtained from the immature eggs. In the case of *Asterias,* however, the egg-water was obtained from maturing eggs. No difference in effectiveness was found between the two solutions.

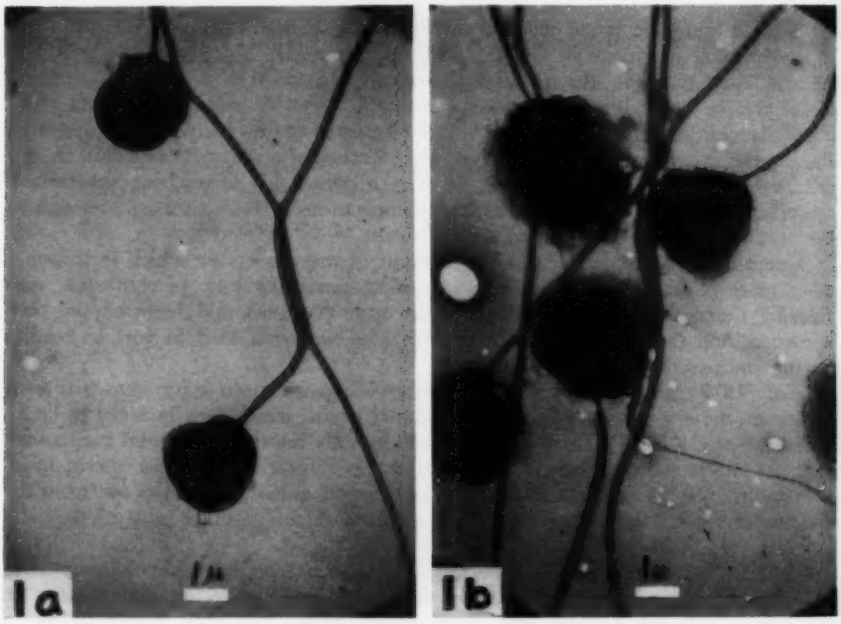


Figure 1. Electron micrographs of *Asterias* spermatozoa fixed in pure sea water, (a) with neutralized formalin; (b) with OsO_4 vapor.

In practice it was found that the jelly of *Asterina* oocytes swells considerably in Ca-low artificial sea water; after having stood for about 30 minutes, such suspensions were centrifuged sufficiently to remove at least part of the jelly, and 4% of 0.36 *M* $CaCl_2$ was added to bring the calcium content approximately to that of normal sea water. In the case of *Asterias,* sufficiently potent egg water was obtained by simply removing and filtering the supernatant fluid from a concentrated suspension of eggs which had stood for at least an hour.

[2] However, there are always a few (less than 10%) of the oocytes in which the germinal vesicles do break down. These fertilize and develop normally.

Spermatozoa were obtained by extirpating the testes, which were kept "dry" in a covered glass container, and the sperm exuding from them was freshly suspended, immediately before use, in the adjuvant solution. Throughout the experiments, crystalline egg albumin, dissolved in sea water and filtered, was used as the adjuvant, in concentrations ranging between 0.1 and 1.0%.

Most of the observations were made on living sperm suspensions, with the oil immersion objective of a phase contrast microscope. For photographing and electron microscopic preparations, the suspensions were fixed with osmium vapor. Because of the presence of the dissolved albumin, however, it proved very difficult to obtain satisfactory preparations for direct electron microscopic observation. This difficulty was partially resolved by making replicas. The electron microscope was a Hitachi Standard, operating on 50 KV.

Results

The head and middle piece of the starfish spermatozoan make up an approximately spherical structure, slightly flattened anteriorly (Fig. 1a). In the living spermatozoan a bluntly cone-shaped acrosome can be seen imbedded in the nuclear

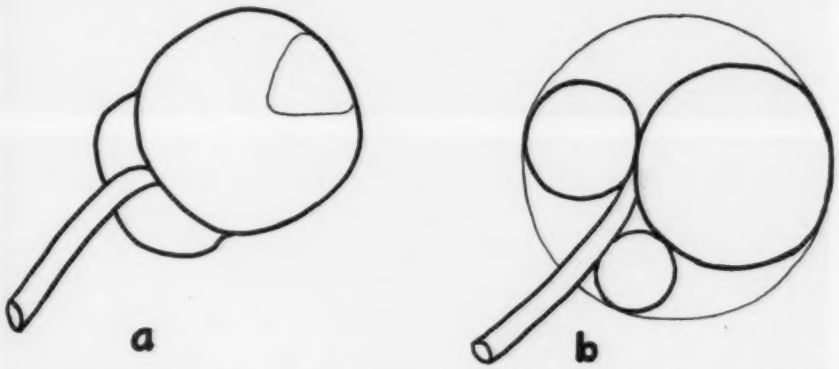


FIGURE 2. (a) Diagram of starfish spermatozoan in sea water. Middle piece is closely applied to posterior part of head; acrosome appears as a rounded cone imbedded in nuclear portion of head. Tail curves around middle piece and extends more or less directly backward. (b) Diagram of moribund starfish spermatozoan, showing nucleus, acrosome and middle piece rounded up separately within inflated membrane.

material so that the apex of the cone points backward, toward the middle piece (Fig. 2a). With phase contrast the acrosome material appears strongly refringent, in contrast to the material of the nucleus. The middle piece is relatively large, and is closely applied to the base of the head. While the combined length of the starfish sperm head and middle piece is only about half that of a moderately-sized sea urchin spermatozoan (*i.e., Hemicentrotus*), the tail is fully as long as any of the sea urchin sperm tails (50 μ). The head and middle piece are enclosed in a single membrane which is not apparent in normal specimens, but becomes visible

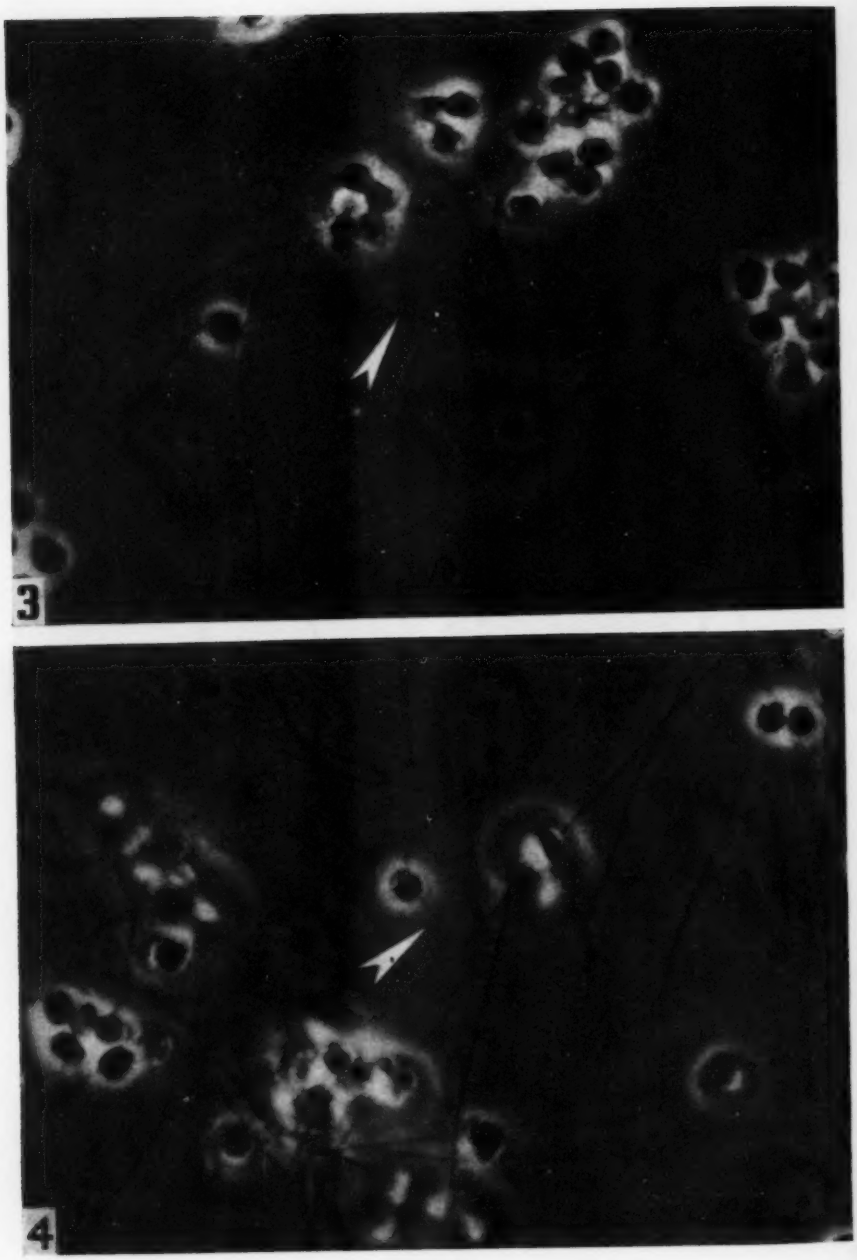


FIG. 3-4.

in moribund sperm or following various types of treatment. In an aged suspension, moribund individuals are frequently seen in which the membrane has separated and become somewhat inflated, and within this spherical membrane the nucleus, middle piece and a refringent body, presumably the acrosome, are all to be seen as discrete spheres (Fig. 2b). The nucleus, middle piece and tail apparently retain their original connection with each other at one point, but the acrosome has moved out of place entirely and seems to be free within the membrane.

As Metz has reported, although starfish sperm are usually not appreciably activated either by suspension in sea water or by addition of homologous egg water, they become intensely active when they are suspended in egg albumin-sea water.

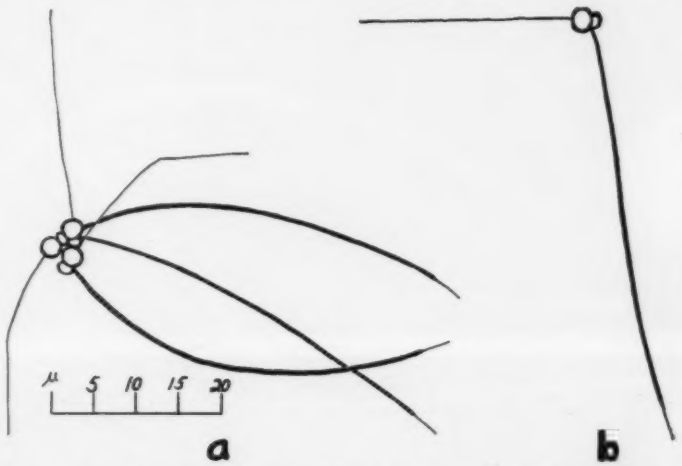


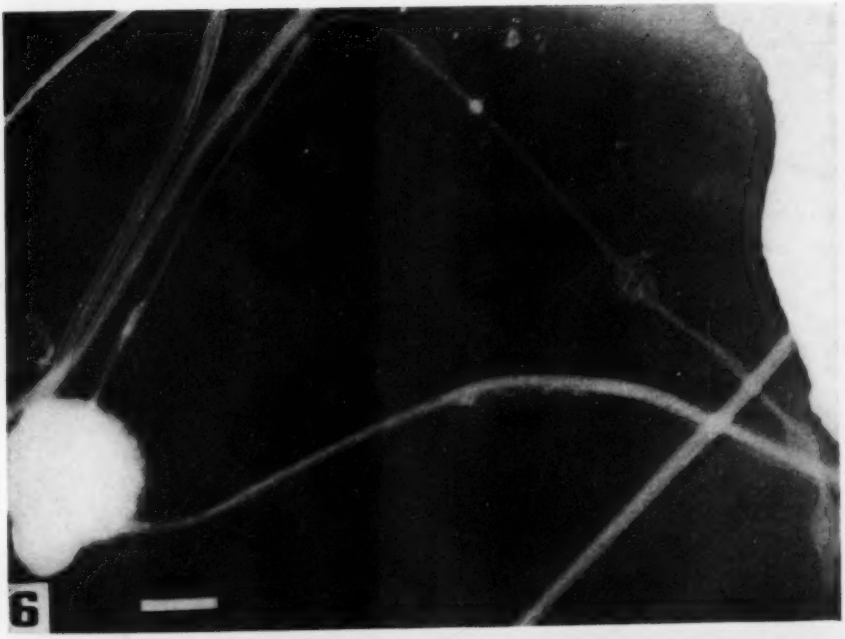
FIGURE 5. Camera lucida drawings of *Asterina* spermatozoa suspended in 0.5% albumin-sea water, after addition of homologous egg-water. (a) Small cluster of agglutinated sperm, showing the acrosome filament on each. A strong current of water was passed across this field from the left; two of the filaments were bent and all the tails swept toward the right side. (b) Single reacted spermatozoan held against cover glass by acrosome filament.

Such activated sperm, however, do not show any change in structure when they are examined either in the living state with phase contrast, or with the electron microscope.

If egg-water is then added to the suspension of spermatozoa in albumin-sea water, and a sample is observed under high magnification, many clumps including from a few to several hundred spermatozoa can be seen, the heads in contact with each

FIGURE 3. Phase contrast micrograph of *Asterina* spermatozoa suspended in 1% egg albumin-sea water, fixed with OsO_4 vapor after addition of homologous egg-water. Most of the spermatozoa are included in small head-to-head clusters; arrow indicates several acrosome filaments stuck to underside of coverglass.

FIGURE 4. Single *Asterina* spermatozoan which has reacted to egg-water focussed to show origin of filament in acrosome region of head.



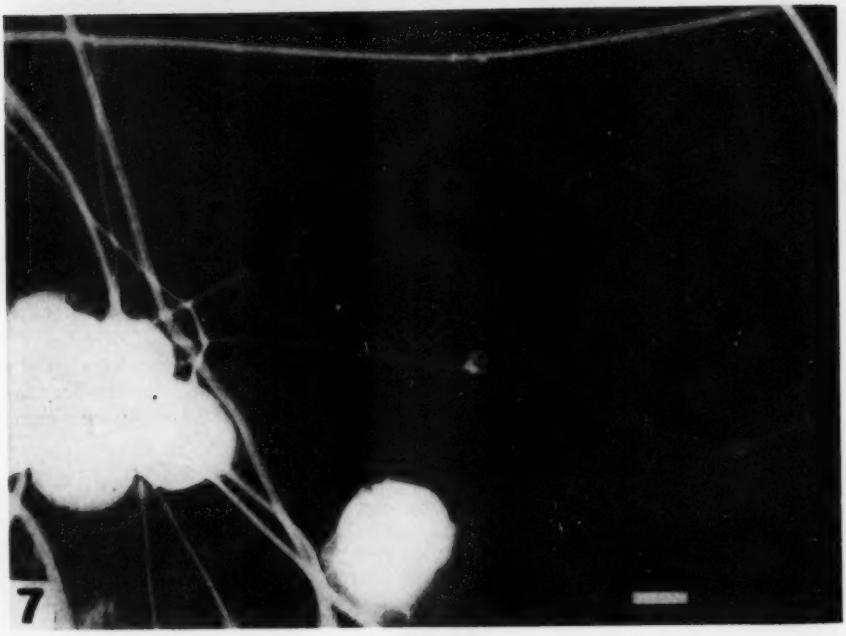


FIG. 6-7.

other, and the tails in vigorous movement (Fig. 3). This agglutination is irreversible.

Many other spermatozoa are in rapid solitary motion, and some can be found with their heads stuck against the glass surfaces, while their tails move freely. Close examination shows that these spermatozoa are held affixed to the glass by a long (ca. 25 μ), very slender filament which extends perpendicularly from the center of the acrosome surface (Figs. 3, 4 and 5a and b, 6). These filaments cannot be seen on swimming spermatozoa, but fixation of the suspension shows that this reaction of the acrosome has taken place in a large proportion of the cells. Especially in the case of the small agglutinated clusters, the filament can be seen on each spermatozoan (Figs. 5a, 7).

Measurements of camera lucida drawings of several of these filaments ranged between 22 and 28 μ. In the living state the filaments show considerable rigidity, bending only partially in the direction of a strong current of water passed over

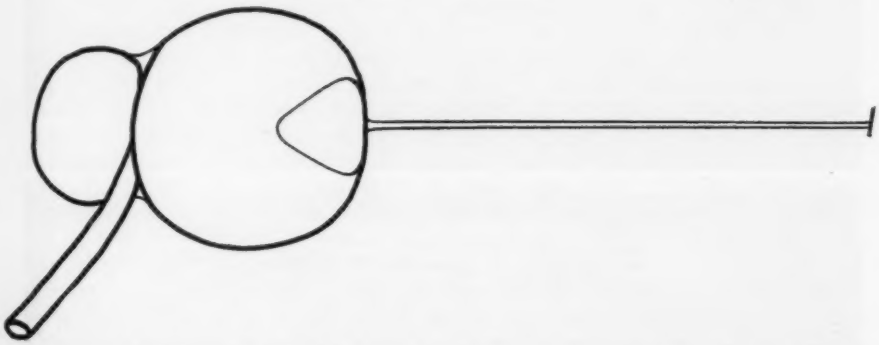


FIGURE 8. Diagram of starfish spermatozoan after acrosome reaction has taken place. Filament extends from center of acrosome surface; middle piece has become nearly spherical, and tail projects laterally, between head and middle piece. Only proximal portion of acrosome filament indicated.

them, and returning to their original position when the current is stopped. They can also be seen to vibrate when hit by a passing spermatozoan, and are sometimes found broken, with part of the filament attached at an angle to the remainder. Some time after the addition of the egg-water, when the actively moving spermatozoa have become sufficiently stationary to permit observation, some of them can be seen to have short fragments of the filaments still projecting from the center of the acrosome surface. Apparently the rest has broken off, during their swimming

FIGURE 6. Shadowed electron micrograph of *Asterina* spermatozoan; suspended in albumin-sea water and mixed with homologous egg-water on collodion membrane; fixed with OsO_4 vapor. Acrosome filament has been bent in handling, and vesicles formed at various points, probably as an effect of the fixative. Note that the membranous sheath of the filament seems to be closed at the tip, and that the central core gives evidence of spiral fibrillar structure.

FIGURE 7. *Asterina;* same treatment as above. Acrosome filaments projecting from agglutinated clump of spermatozoa.

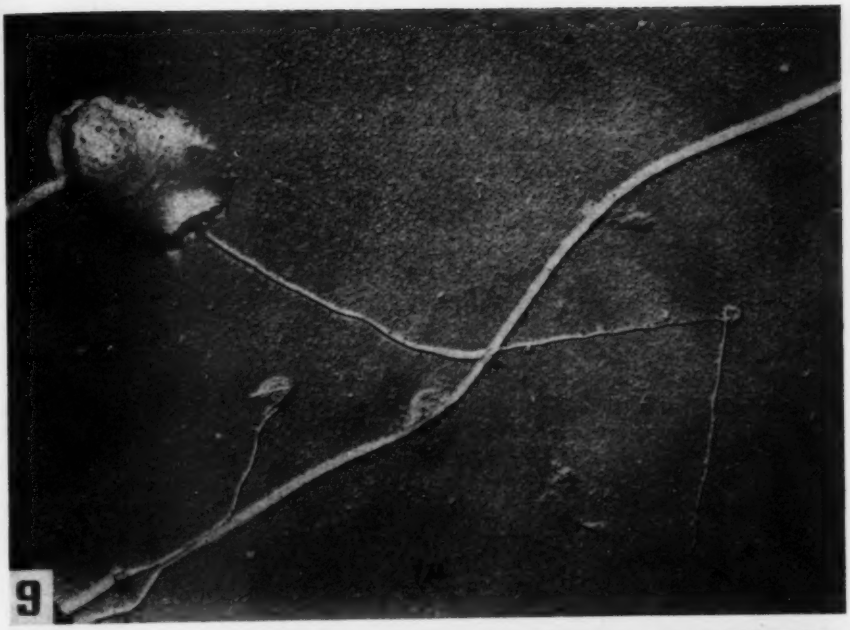
about. Whether the filaments gradually disintegrate in sea water could not be determined definitely for this reason; it is certain that those which have been stuck to the glass from the beginning remain apparently unchanged for at least 30 minutes, but these are not fully exposed to a possible dissolving action of the sea water. No acrosome filaments could be seen on spermatozoa fixed with formalin, although they were well preserved after fixation with osmium vapor. This latter fixative, however, was quite ineffective with respect to the nuclear part of the sperm head, which became extremely flattened and diffuse in outline when the preparation was dried (Figs. 1b, 6, 7, 9, 10).

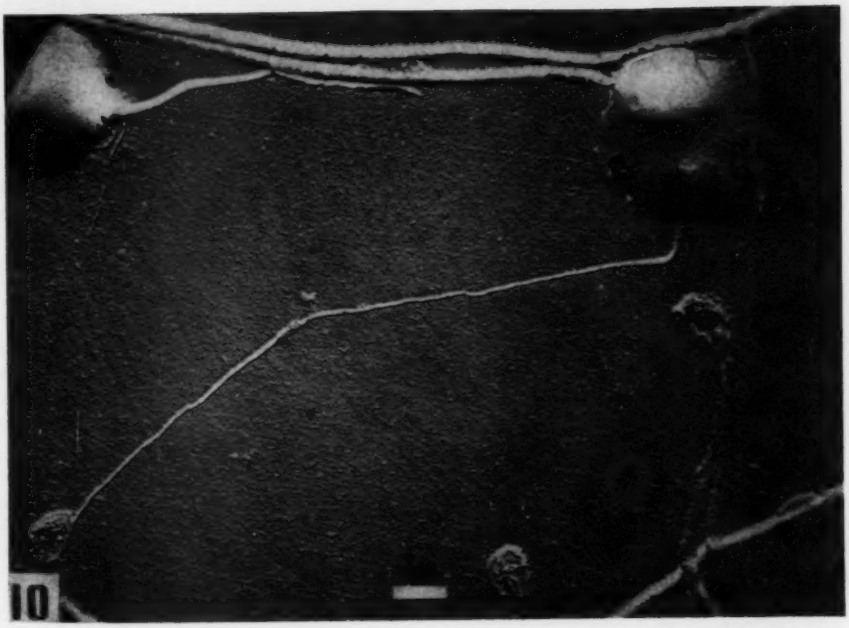
Besides the appearance of the acrosomal filament, there is a structural readjustment which takes place in response to the stimulus of egg-water and makes it possible to differentiate between spermatozoa which have reacted and those which have not. This consists in what appears to be a partial relaxation of the tight enveloping membrane, so that the middle piece rounds up and is less closely applied to the posterior part of the nucleus (Fig. 8; compare with Fig. 2a). Moreover, the tail, which in untreated spermatozoa curves around the middle piece before extending straight backward, now projects laterally from the posterior midpoint of the head (see also Figs. 5a and b, 6, 7, 8, 9, 10). This phenomenon was reported by Chambers (1930), who mentions an "impression that the head of the sperm is bent to one side" (p. 352), and further says (p. 352), "Occasionally a spermatozoan appears to be carried through the jelly with the base of its tail at right angles to the attachment of the insemination filament, while the rest of the tail is curved so as to trail behind." With phase contrast (dark contrast), the reacted acrosome region is no longer brightly refringent, appearing grayish and darker than the nuclear material.

Concerning the fine structure of the acrosome filament, not much can be said with certainty on the basis of the available electron micrographs. It appears to have a slightly greater diameter at the base than at the tip, although this difference cannot be detected in living specimens. There is definitely a central core, probably fibrous, surrounded by a sheath or membrane (Fig. 6). In fixed specimens, local blister-like swellings of this membrane are often found (Figs. 6, 7, 9). The end of the filament apparently has no special structure (Figs. 7, 9), although Figure 6 indicates that the tip is enclosed by the membrane. The knob-like condition of the filament tip in Figure 10 is apparently the result of fixation, and has no particular significance with respect to the acrosome filament itself, since the axial filaments at the ends of the sperm tails in the same preparation also reacted in the same way.[8] This at least suggests that the two slender filaments may have certain properties in common. They are further similar in that both appear to be rather sticky, since spermatozoa are often held firmly to the glass surfaces by the two filaments, while all the other parts—the head, middle piece and sheathed part of the tail—are movable against the glass.

In no case, in either *Asterina* or *Asterias*, has the acrosome reaction been found to take place in the great majority of the spermatozoa, as it does in sea urchins. Most of the sperm which fail to react have small, spherical heads, with the middle piece closely applied to the base of the head; while those which react to egg water

[8] These artifacts are no doubt identical with the vesicles formed at the tips of silver salmon sperm tails on fixation with osmium tetroxide and Zenker's fluid (Lowman, 1954).





FIGURES 9 AND 10. Replicas taken from *Asterias* spermatozoa suspended in 0.1% albumin-sea water, and fixed with OsO_4 vapor after addition of homologous egg-water.

are larger, the anterior part of the head, consisting of the acrosome surface, is flattened, and the middle piece is broad and prominent and its junction with the head is clearly differentiated. It seems possible that the spermatozoa of the smaller type may be non-functional.

Preliminary observations of fertilization in the three starfish species under consideration show that when insemination is carried out soon after the extrusion of the first polar body (see Delage, 1901), the eggs fertilize monospermically and develop normally, at least in the early stages. In all three species, within the minimum of 20–30 seconds which is required for insemination and adjustment of high dry (phase contrast) focus, the inseminating spermatozoan has become attached to the vitelline membrane by a very slender filament and is moving steadily through the dense jelly layer. About the time it reaches the vitelline membrane, this is being pushed away from the egg surface by a low fertilization cone. The spermatozoan traverses the intervening membrane and can be seen momentarily within the hyaline protoplasm of the cone, which becomes somewhat larger but is usually restrained during its developing phase by the very slowly rising fertilization membrane (see Dan, 1950, Fig. 7). By the time the sperm tail is nearly inside the egg, the membrane has lifted away from the degenerating cone, which rather quickly subsides in an irregular fashion and disappears completely within 6–10 minutes after insemination.

Discussion

The fact that starfish spermatozoa can be shown to produce, in response to the stimulus of species egg-water, a long, slender filament which coincides in appearance with the filament supposed by various workers to proceed from the egg surface, necessitates a reconsideration of the evidence on which this supposition was based. Chambers (1930) cites Fol as having rejected the possibility that the filament originated in the spermatozoan because he was unable to detect any diminution in the size of the heads of spermatozoa attached to such filaments. For the following several reasons the decision seems unavoidable that Fol was mistaken in drawing such a conclusion:

1. the heads of starfish sperm are far from uniform in size, as observed in the living state, under phase contrast oil immersion;
2. estimates from observations of living sperm and electron micrographs indicate that the diameter of the living starfish sperm head is about 2 μ, which is too small, even with modern optical equipment, to permit accurate estimation of minor changes in volume;
3. calculations based on measurements of electron micrographs of osmium-vapor-fixed sperm heads and acrosome filaments indicate that loss of the volume of substance contained in a filament 25 μ in length and 0.13 μ in diameter would result in a reduction of the sperm head diameter of only about 2%;
4. the partial separation and rounding-up of the middle piece and main part of the sperm head at the time of the acrosome reaction produce sufficient change to obscure a much greater loss of substance than that necessary to produce the filament.

Fol's description of the fertilization process in echinoderms was so detailed and accurate that, as Chambers pointed out when he undertook a re-examination of the question nearly half a century later, "even recent textbooks comment upon the completeness of his observations" (1923, p. 821). Although Chambers referred to Fol's idea of the mechanism in starfish eggs, by which "the presence of spermatozoa in the immediate vicinity causes the egg to respond by forming on its surface a conical elevation which attracts the spermatozoan from a distance" as "rather extraordinary" (loc. cit.), he followed Fol in this interpretation, apparently in lieu of sufficient evidence to the contrary.

Chambers found that when starfish eggs are in the optimum condition for fertilization, the process takes place so rapidly that the filament is already extending between sperm head and cone tip by the time the earliest observation can be made. Relying on Fol's assumption of the "attraction cone" as the site of origin of the filament, Chambers records a number of observations in this and his later paper (1930) which convincingly serve to demonstrate its existence and show that it is the agency by which the spermatozoan is drawn through the jelly to the egg surface. However, the best evidence which he could muster, in the course of the two studies, for the origin of the filament in the attraction cone depends on the assumption that sperm entrance takes place in the same manner whether the eggs are in the optimum condition or either under- or over-mature.[4]

It is well known that in sea urchin eggs there are highly significant differences between the modes of sperm entrance, and more especially between the reactions of the eggs to insemination, under different conditions of maturity. In immature eggs there is apparently no block except spatial limitation to the entrance of any number of sperm; no fertilization membrane is raised; and the only apparent response of the egg cytoplasm to sperm entrance is the formation of conspicuous, semi-permanent structures on the surface which appear to be bundles of protoplasmic fibrils and bear little resemblance to the small, rounded, temporary cones of normal fertilization. Hobson (1927) found that in the oocytes of *Asterias rubens*, which had the germinal vesicles still intact and were "never observed to cleave or to complete their maturation" (p. 100), the reaction to sperm entrance was so similar to that reported by Seifritz (1926) in oocytes of *Echinarachnius parma* that he did not bother to repeat the description.

Since the optimal condition in sea urchin and sand dollar eggs lasts for a very long period, the various changes in the fertilization reaction associated with the passage of time may equally well be the result of a senescent loss of vitality, as of a condition of "over-maturity" such as that occurring in "post-optimal" starfish eggs, and there is probably little profit in attempting to compare the two sets of phenomena.

Just, on the other hand, attacked the interpretation of Fol and Chambers on the basis of his own observations of starfish eggs, which, he wrote, "indicate that maturated eggs lose the capacity for normal fertilization, and parallel with this loss runs the production of filaments." ". . . the production of filaments by *Asterias* ova . . . as a response to insemination is a phenomenon quite apart from the behavior

[4] The terms "under-" and "over-mature" are used with reference to the "optimum condition for fertilization," which lasts only during the period between the extrusion of the first and second polar bodies.

in the normal fertilization reaction" (Just, 1929, p. 319). *"Fol used stale eggs,* which accounts for the filament formation" (op. cit., p. 322).

The observations of other workers, then, do not support Chambers' primary assumption, that the fertilization reaction follows the same process regardless of the state of the egg with respect to the meiotic divisions. As a result, his conclusion based on this assumption, that since filaments have been observed to arise from the entrance cones in immature eggs and in old eggs, the filament in normal fertilization must necessarily originate in the same way, loses much of its validity.

With respect to eggs in the optimum condition for fertilization, Chambers says, "In fresh maturing eggs I have never been able to see the cone without also seeing the advancing sperm and the filament connecting the two. The formation of the filament is apparently too rapid" (1930, p. 354). This is the fact which remains when we cancel out of Chambers' observations the influence of Fol's dictum that the fertilization filament could not have arisen from the spermatozoan.

On the other hand, while Just insists that a strand which he observed connecting the sperm head with the fertilization cone "is a prolongation of the spermatozoon" (op. cit., p. 321), the fact that he based his observations partly on fixed material automatically raises a question as to their value. It seems obvious that such a process as the approach of a spermatozoan through the jelly to the egg surface cannot be preserved in successive stages by any of the ordinarily used methods of fixation, since they either cause the jelly layer to shrink or dissolve it completely away. In the best case the exact relation of the sperm to the egg surface could only be captured by fixation after the spermatozoan had become firmly attached to the egg cytoplasm.

At least the first part of Just's observation seems to have been made on living material: "The intensely active spermatozoa rush toward the jelly hull; of these, one rapidly moving through it reaches the egg within 5 seconds after insemination" (loc. cit.). In the experience of Chambers, as well as of the writer, it has been found that at least twenty or thirty seconds are required to bring the freshly inseminated eggs into high power focus and discover one with a fertilizing spermatozoan attached exactly in the largest optical section. The sperm is by this time already connected with the egg surface by the filament in question, and according to Chambers' figures (1923, Fig. 2, a-c; 1930, Fig. 2, A-H), about one minute more is required for the sperm to pass through the jelly layer and reach the surface of the vitelline membrane. Preliminary observations by the writer on *Asterias amurensis* showed the sperm head reaching the membrane surface about two minutes after insemination (at 17° C.). In *Asterina pectinifera,* the sperm passed through the narrow jelly layer within 50–60 seconds after insemination (23° C.). *Astropecten scoparius* has a somewhat wider jelly layer, which the sperm crossed in about seventy seconds (at 25° C.).

A more detailed study of the fertilization reaction in these three species will be presented in a later paper; these preliminary data are cited as the basis of the writer's inability to accept Just's figure of five seconds as the time required for a spermatozoan to traverse the jelly layer of the *Asterias* egg.

Just further describes the process as he saw it in both living and fixed preparations: "As the cone grows the spermatozoon is pushed off from the egg, a delicate strand connecting the tip with the apex of the cone. This strand never attains the

length given by Fol, and of course could never therefore be equal to the greater length figured by Chambers" (1929, p. 321). Just does not explain how or why such a structure is produced, beyond stating categorically, *"this strand is a prolongation of the spermatozoon, the tip of which is fixed within the cone"* (Just's italics; op. cit., p. 321). In the conclusion of his paper, however, he draws a comparison which probably reveals the source of his confidence in making the above statement: "This cone in *Asterias* egg closely resembles that in *Nereis* egg," except for the time factor. "Moreover, the strand between the sperm head and the cone in both cases has the same origin, namely, from the spermatozoon itself" (op. cit., p. 324).

It seems doubtful whether this analogy provides a sufficiently firm basis for a flat rejection of Chambers' observations as well as his interpretation.

Since Hörstadius, in describing fertilization in *Astropecten aranciacus,* does not mention filaments of the order of size of those under consideration, a discussion of his interpretation of the process is not strictly appropriate at this time. However, since the writer has clearly observed the formation of acrosome filaments in response to egg-water in the spermatozoa of *Astropecten scoparius,* and also in the fertilization process, it appears likely that in *A. aranciacus,* also, filaments from the sperm acrosome give the stimulus for the elevation of the cylindrical cones observed by Hörstadius. That author himself suggests that the formation of very many large cones is a characteristic response of immature eggs to insemination.

To summarize this discussion concerning the origin of the "fertilization filament"—it appears that, among later workers, only Chambers actually observed the filaments first discovered by Fol. Just's categorical statement quoted above with respect to the length of the filament, as well as the sequence of steps in its formation and their timing, all indicate that he failed completely to see the structure described in the earlier papers. Moreover, since Chambers, in spite of his accurate observation, was unable to establish conclusively the origin of the filament in the "attraction cone," we find the whole weight of this explanation of starfish fertilization resting upon Fol's assumption. This, in turn, depends upon his belief that he should have been able to observe a difference in the size of sperm heads before and after the extrusion of the filament.

If the criticisms of this assumption listed above be allowed, it follows that no incontrovertible evidence has been presented proving that the filament, observed by Fol, Chambers and the writer connecting the sperm head with the egg surface, arises from the egg. There is, moreover, no other case in animal fertilization, so far as the writer is aware, in which an egg has been shown to reach out and capture a spermatozoan.

On the positive side, evidence has been found (unpublished data) to show that the acrosomes of sea urchin and sand dollar spermatozoa undergo a reaction, in response to the stimulus of species egg-water, which can most readily be interpreted as providing a method of penetrating the first serious obstruction [5] which the spermatozoa encounter in their progress toward the egg pronucleus—the vitelline membrane. Since the tip of the sperm head is already in contact with the membrane, the liberation of a lytic substance, together with the reaction of the egg

[5] In these species, the spermatozoa swim through the jelly layer in a radial direction with at most only a slight reduction in speed.

cytoplasm would presumably be sufficient to effect penetration. In the case of the starfish egg, on the other hand, an impenetrable jelly layer intervenes between the spermatozoan and the living egg surface. This necessitates the extrusion of a projection of the acrosome, long enough to cross the jelly layer, and sufficiently rigid to penetrate the vitelline membrane and establish contact with the reactive cytoplasm. It is believed that these conditions are fulfilled by the filament which is extruded from the starfish acrosome in response to the presence of species egg-water.

At present there is no evidence as to how the filament is drawn into the egg cytoplasm. That such a process is entirely possible is shown by the fact that in the sea urchin, *Mespilia globulus*, the entrance of the sperm tail may take place very slowly (Dan, 1950), so that the sperm aster appears and even syngamy is complete before the tail is more than halfway into the egg. In this case it is impossible to imagine that the motionless tail is proceeding under its own motive power; nevertheless, its whole length is taken into the cytoplasm within about 15 minutes.

Tyler (1948) has suggested an explanation of the starfish filament in terms of a fertilizin-antifertilizin interaction, in which active antifertilizin groups on the sperm would combine with active fertilizin groups on the micelles of the jelly, resulting in precipitation, or contraction, of the micelles. Since these are assumed to be anchored to the surface of the egg, their contraction would draw the spermatozoan a slight distance toward the egg and into contact with new micelles, where the reaction would be repeated, until the sperm head finally reached the egg surface.

This is an ingenious attempt to fit the starfish fertilization process into the general echinoderm pattern, and represents a decided advance beyond the appeal to a mysterious force directing an attraction cone filament to a waiting spermatozoan. Observation of the actual fertilization process shows, however, that the filament has already attained its full diameter while the sperm head is still nearly the whole width of the jelly layer away from the egg surface, and no change in this diameter can be detected as the sperm head approaches the egg. Moreover, it seems questionable whether a filament of microscopically observable dimensions could be produced by the mechanism suggested, even given a radial arrangement of the submicroscopical jelly micelles. Finally, it must obviously be quite hopeless to rely on this scheme for an explanation of the filaments found on egg-water-treated spermatozoa.

The abrupt extension of a long, slender filament is a phenomenon already familiar in the discharge of trichocysts by ciliates and nematocysts by the coelenterates. Since the acrosome of the starfish spermatozoan measures only a fraction of a micron, it seems hardly possible that any very complex mechanism could be contained within it. This is one consideration in favor of a trichocyst-like discharge mechanism. However, the problem is within the range of direct experimental attack, and further discussion will be reserved until evidence is available.

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Summary

1. When the spermatozoa of three starfish species, *Asterina pectinifera, Asterias amurensis* and *Astropecten scoparius,* are suspended in a dilute (0.1–1.0%) solution of egg albumin in sea water and mixed with homologous egg-water, three effects can be noted:

a. many of the spermatozoa are agglutinated by their heads, forming permanent clusters;
b. from the center of the acrosome of each agglutinated spermatozoan there has been extended a long (ca. 25 μ), very slender, straight filament which possesses considerable rigidity;
c. in all the spermatozoa which have so reacted, there is a rearrangement of the principal parts, so that the middle piece is less tightly apposed to the head and the tail appears to be inserted laterally, between the head and middle piece.

2. A critical examination of the widely accepted explanation of starfish fertilization—that the effective spermatozoan is drawn through the jelly layer to the egg surface by a filament originating in an "attraction cone"—shows that this depends mainly upon an assumption made by Fol, which various considerations show to be of doubtful validity. In the light of the fact that starfish spermatozoa produce a similar filament from their acrosomes, on contact with dissolved jelly substance, it is proposed that this sperm acrosome reaction is the source of the filament which, extending through the jelly, stimulates the egg cortex. Following this stimulation, the egg cytoplasm draws in the filament with the attached spermatozoan and simultaneously forms a fertilization cone beneath the vitelline membrane, which separates as the fertilization membrane. This sequence of events is the same as that constituting the fertilization reaction in other echinoderms.

LITERATURE CITED

Chambers, R., 1923. The mechanism of the entrance of sperm into the starfish egg. *J. Gen. Physiol.*, **5**: 821–829.

Chambers, R., 1930. The manner of sperm entry in the starfish egg. *Biol. Bull.*, **58**: 344–369.

Dan, J. C., 1950. Sperm entrance in echinoderms, observed with the phase contrast microscope. *Biol. Bull.*, **99**: 399–411.

Dan, J. C., 1952. Studies on the acrosome. I. Reaction to egg-water and other stimuli. *Biol. Bull.*, **103**: 54–66.

Delage, Y., 1901. Etudes expérimentales sur la maturation cytologique et sur la parthénogénèse artificielle chez les echinodermes. *Arch. de Zool. exp. et gén.*, **9**: 285–326.

Fol, H., 1877. Sur le commencement de l'hénogenie chez divers animaux. *Arch. de Zool. exp. et gén.*, **6**: 145–169.

Glaser, O., 1914. A quantitative analysis of the egg secretions and extracts of Arbacia and Asterias. *Biol. Bull.*, **26**: 367–386.

Hobson, A. D., 1927. A study of the fertilization membrane in the echinoderms. *Proc. Roy. Soc. Edinb.*, **47**: 94–117.

Hörstadius, S., 1939. Über die Entwicklung von *Astropecten aranciacus* L. *Pubbl. Statz. Nap.*, **17**: 221–312.

Just, E. E., 1929. The production of filaments by echinoderm ova as a response to insemination, with special reference to the phenomena exhibited by ova of the genus *Asterias*. *Biol. Bull.*, **57**: 311–325.

JUST, E. E., 1930. The present status of the fertilizin theory of fertilization. *Protoplasma*, **10**: 300–342.

LOEB, J., 1914. Cluster formation of spermatozoa caused by specific substances from eggs. *J. Exp. Zool.*, **17**: 123–140.

LOWMAN, F. G., 1954. Electron microscope studies of silver salmon spermatozoa (*Oncorhynchus kisutch* (Walbaum)). *Exp. Cell. Res.*, **5**: 335–360.

METZ, C. B., 1944. Agglutination of starfish sperm by homologous egg water. *Anat. Rec.*, **89**: 559.

METZ, C. B.. 1945. The agglutination of starfish sperm by fertilizin. *Biol. Bull.*, **89**: 84–94.

NOMURA, S., 1926. On the agglutinating substance of the egg of the starfish, *Asterina pectinifera*. *Sci. Rep. Tohoku Imp. Univ.*, **I**: 83–87.

SEIFRITZ, W., 1926. Protoplasmic papillae of Echinarachnius oocytes. *Protoplasma*, **1**: 1–14.

TYLER, A., 1941. The role of fertilizin in the fertilization of eggs of the sea-urchin and other animals. *Biol. Bull.*, **81**: 190–204.

TYLER, A., 1948. Fertilization and immunity. *Physiol. Rev.*, **28**: 180–219.

WOODWARD, A. E., 1918. Studies on the physiological significance of certain precipitates from the egg secretions of Arbacia and Asterias. *J. Exp. Zool.*, **26**: 459–497.

COMPARATIVE STUDY OF THE GILL AREA OF MARINE FISHES

I. E. GRAY

Department of Zoology, Duke University, Durham, N. C.

Among fishes there are obvious species differences in number of gill arches, as well as in number and length of gill filaments. Less obvious are species differences in number and size of the respiratory units, the gill lamellae. This suggests that there is also variation in gill surface area per unit of size of fish. Since fishes occupy a variety of ecological niches it is of interest to know if the gill area may in any way be correlated with activity and habitat.

Although there have been many studies on the anatomy of fish gills, only a few investigators have concerned themselves with gill area. Riess (1881) was among the first to attempt accurate measurements of gill surface. He found that pike had 125 sq. mm. of respiratory surface per gram of body weight. Putter (1909) determined gill surface area of a few fishes, particularly *Scorpaena*, and felt that the respiratory surface was proportional to body surface but not to body weight as maintained by Riess. The most extensive study on a single species was made by Price (1931), who followed the changes in gill area throughout the development of small-mouthed bass. The only study of a comparative nature appears to be that of Schöttle (1931), who found a reduction of gill surface in terrestrial gobiiform fishes compared to strictly aquatic species.

The studies reported here were carried out exclusively on marine teleost fishes representing twenty-three families and thirty-one species. The work was done in part at the Marine Biological Laboratory, Woods Hole, Mass., and in part at the Duke University Marine Laboratory, Beaufort, N. C. Partial support came from the Duke University Research Council.

For permission to participate in a cruise of the *Albatross* in North Carolina waters when several dolphin were obtained, and for use of laboratory space during part of one summer, grateful acknowledgment is made to the Institute of Fisheries Research of the University of North Carolina and to the U. S. Fish and Wildlife Station at Beaufort, respectively.

Methods

Each gill filament is made up of numerous respiratory units, the lamellae or platelets. To obtain the respiratory area of a fish it is necessary to know the total area of the lamellae. It is, of course, impractical to measure each of the thousands of lamellae present; consequently a sampling method must be employed.

After weighing the fish and making outline drawings for later determination of body surface area, the gill arches of one side were dissected out, carefully separated, and placed in dishes of sea water, one arch to each dish. The number of filaments on each side of each arch was counted under a dissecting microscope. The average length of the filaments was determined by measuring every tenth filament with

vernier calipers. From these measurements the average length of the filaments was established. By placing filaments of approximately average length under the low power objective of a compound microscope and using either a stage or an ocular micrometer, the number of lamellae per millimeter of filament was determined. What appeared to be lamellae of average size were separated from filaments of average size and placed on slides. Camera lucida drawings were then made of several lamellae. Usually this could be done using the lower power objective of a compound microscope. Planimeter readings were made of the camera lucida drawings and an average taken.

Knowing the number of gill filaments, the average length of the filaments, and the number of lamellae per millimeter of filament, the total number of gill platelets was readily determined. From this and the known magnification and area of the camera lucida drawings, the total area of gill surface could be computed.

In those species with extremely delicate filaments, the task of obtaining undamaged platelets was facilitated by hardening the filaments for a few minutes in formaldehyde or Bouin's fluid. The central cartilaginous support of the filament that runs through each lamella was not included in the computation of area. Since the lamellae are functional on both sides the determined area was doubled.

Admittedly, with so many manipulations, there is a large possibility of error in determining gill area. However, all specimens were treated in the same manner so that the results obtained would be comparable.

Body surface area of most species was found by drawing around the fish, allowing for body thickness and omitting dorsal, anal and paired fins, and determining the area of the outline with a planimeter. This method is quite adequate for depressed and compressed fishes, such as goosefish and butterfish. It can be used with more difficulty with odd-shaped fishes like sea robins, puffers, and toadfish. The body surface areas of these latter species were also determined by the more laborious method of covering the body with pieces of paper and making planimeter readings on each piece. The use of a general formula for determining body surface area was discarded because the different species differed in form and weight and a correction factor for each species would first have to be obtained. This has been discussed in a previous paper (Gray, 1953).

Results and Discussion

In Table I, which includes 31 species representing 23 families of marine teleosts, it can be clearly seen that species differ widely in number of respiratory lamellae and in respiratory area. This is true whether comparison is based on unit of body weight or on unit of body surface. In this table species are arranged in descending order of gill area per square centimeter of body surface. The contrast in respiratory area of those species near the top when compared with those near the bottom is great. One familiar with common marine fishes of the Atlantic coast will see a general correlation between respiratory area and species activity.

For convenience of discussion, and with no sharp demarcation between them, the fishes may be divided into three groups.

I. Active, schooling, migrating, fast swimming, streamlined fishes that, at least in the adult stage, seldom frequent the smaller estuaries. To this group clearly belong the mackerels, menhaden, dolphin, bluefish. At least during the summer

months these species are in constant motion as they feed on plankton or follow schools of smaller fish.

II. Fishes of moderate activity, estuarine species limited in their daily travels. These include such species as scup, sheepshead, sea bass and others that hang around jetties, wrecks, piers, etc., feeding on crustaceans, molluscs, and sessile animals. On the lower fringes of this group may be included tautog, sea robins and puffers that spend part of their time resting on the bottom.

III. Relatively sluggish species more or less adapted for benthic life. Toadfish, goosefish, and flounders are good examples.

TABLE I

Gill area of marine fishes

Species of fish	No. of determinations	Average weight (gms.)	Lamellae per mm. of filament	Gill Area (sq. mm.)					
				Per gram of body weight			Per sq. cm. of body surface		
				max.	min.	ave.	max.	min.	ave.
False albacore, *Gymnosarda alleterata*	1	5216	33	1939	1939	1939	4854	4854	4854
Menhaden, *Brevoortia tyrannus*	12	613	29	2547	1241	1773	2704	1300	1828
Dolphin, *Coryphaena hippurus*	4	4015	26	965	618	710	1334	971	1169
Bonito, *Sarda sarda*	2	2192	31	631	558	595	1237	1073	1155
Bluefish, *Pomatomus saltatrix*	1	1035	29	652	652	652	841	841	841
Common mackerel, *Scomber scombrus*	15	182	31	1532	802	1158	1103	543	838
Spanish mackerel, *Scomberomorus maculatus*	2	478	29	770	768	769	731	724	728
Jumping mullet, *Mugil cephalus*	9	166	27	1105	760	954	845	538	654
Hard tail, *Caranx crysos*	3	129	39	1048	894	982	610	567	594
Striped bass, *Roccus lineatus*	4	3059	19	426	148	302	818	415	592
Sheepshead, *Archosargus probatocephalus*	6	2366	19	467	212	328	553	333	488
Bur fish, *Chilomycterus schoepfi*	2	316	13	449	425	437	492	482	487
Scup, *Stenotomus chrysops*	7	395	26	623	391	506	612	352	478
Tautog, *Tautoga onitis*	6	580	18	519	293	392	531	317	435
Red-winged sea robin, *Prionotus strigatus*	11	460	20	768	318	483	642	191	424
Butterfish, *Poronotus triacanthus*	9	199	31	817	386	598	572	252	411
Sea trout, *Cynoscion regalis*	6	807	27	593	221	373	671	253	410
Rudderfish, *Palinurichthyes perciformis*	6	199	24	658	377	506	550	268	388
Remora, *Echeneis naucrates*	2	393	22	783	314	549	—	—	—
Puffer, *Spheroides maculatus*	13	250	16	956	324	470	585	232	372
Sea bass, *Centropristis striatus*	4	244	21	653	439	458	480	233	361
Goosefish, *Lophius piscatorius*	3	6392	11	267	116	196	360	245	299
Harvest fish, *Peprilus alepidatus*	2	71	26	526	483	505	257	230	244
Conger eel, *Leptocephalus conger*	2	2560	19	136	134	135	224	202	213
Brown sea robin, *Prionotus carolinus*	2	213	20	394	326	360	225	197	211
Cutlass fish, *Trichiurus lepturus*	3	116	31	627	361	536	244	138	198
Common eel, *Anguilla rostrata*	4	428	19	398	183	302	236	125	193
Fluke, *Paralichthys dentatus*	5	766	19	328	206	242	225	134	176
Flounder, *Pseudopleuronectes americanus*	2	734	18	218	183	200	134	115	125
Toadfish, *Opsanus tau*	58	233	11	362	94	197	226	69	125
Sand flounder, *Lophopsetta maculata*	1	411	20	188	188	188	90	90	90

The numbers of lamellae per mm. of gill filament are also included in Table I. Here again it is found that, in general, the most active fishes have smaller lamellae placed closer together than do the more sluggish species. Usually a large number of small lamellae means more surface than a small number of large lamellae. But of course other factors such as the length of the filaments, the number of filaments and the number of gills are also important in determining the amount of gill surface.

Fishes with large lamellae spaced far apart often live longer out of water than those with closely packed fine lamellae. A toadfish will live for hours on the laboratory floor; a butterfish dies in a matter of minutes. Delicate closely spaced lamellae adhere together when removed from an aquatic medium and the func-

tional surface is thus greatly reduced. It is the sluggish fishes with low metabolism that have the widely spaced lamellae.

In gobies, Schöttle (1931) has shown that those capable of remaining out of water have gill lamellae so arranged as not to collapse when the fish is on land.

Criteria for estimating degree of activity are unfortunately not based on oxygen consumption. It is desirable but difficult to obtain comparative metabolism data on marine fishes. From necessity most data are obtained from sluggish and moderately active species. Active species are hard to keep in captivity, or even to get to the laboratory, without becoming partially asphyxiated. Some, and this has been observed particularly in the family Scombridae, apparently die quickly from nervous exhaustion. This is especially true of schooling fishes when separated from their companions. However, the fact that it is difficult to maintain these fishes in the

TABLE II

Order of rank of marine fishes based on number of lamellae and on gill area

Gill lamellae per mm. of filament		Gill area per gram of body weight (sq. mm.)		Gill area per cm. of body surface (sq. mm.)	
1. Mackerel	31	1. Menhaden	1773	1. Menhaden	1828
2. Butterfish	31	2. Mackerel	1158	2. Dolphin	1169
3. Menhaden	29	3. Mullet	954	3. Mackerel	838
4. Mullet	27	4. Dolphin	710	4. Mullet	654
5. Sea trout	27	5. Butterfish	598	5. Striped bass	592
6. Dolphin	26	6. Scup	506	6. Sheepshead	488
7. Scup	26	7. Rudderfish	506	7. Scup	478
8. Rudderfish	24	8. Sea robin	483	8. Tautog	435
9. Sea bass	21	9. Puffer	470	9. Butterfish	429
10. Sea robin	20	10. Sea bass	458	10. Sea robin	424
11. Striped bass	19	11. Tautog	392	11. Sea trout	410
12. Sheepshead	19	12. Sea trout	373	12. Rudderfish	388
13. Eel	19	13. Sheepshead	328	13. Puffer	372
14. Fluke	19	14. Striped bass	302	14. Sea bass	361
15. Tautog	18	15. Eel	302	15. Eel	193
16. Puffer	16	16. Fluke	242	16. Fluke	176
17. Toadfish	11	17. Toadfish	197	17. Toadfish	125

laboratory is at least a qualitative indication of relative activity even though not a quantitative one. It is these species that have the greatest relative gill surface.

Researches of others on some of the same species lend support to the theory that the active fishes have higher metabolism than bottom dwellers. Hall and Gray (1929) in their hemoglobin studies showed that the mackerel with 43 and the menhaden with 41 were relatively high in mgs. of iron per 100 cc. of blood, in contrast to the goosefish with 14.7, toadfish, 13.5, and sand flounder, 11.5. Scup (25.3), sea robin (23.7), and puffer (21.5) were intermediate. Also, Hall and McCutcheon (1938) have pointed out a correlation of hemoglobin function with activity and habitat in marine fishes. Mackerel and toadfish represented the extremes of the fishes they studied, with scup occupying an intermediate position.

Similarly, Gray and Hall (1930) showed that active fishes had greater amounts of blood sugar per 100 cc. of blood than did less active species (menhaden 75 mgs., mackerel 64, scup 53, sea robin 37, puffer 23, toadfish 15, and goosefish 6).

Vernberg and Gray (1953) found that a correlation exists between oxygen consumption of excised brain tissue and activity in fishes. The O_2 consumption of menhaden brain was found to be nearly twice that of toadfish brain, with moderately active fishes occupying an intermediate position.

Thus it seems that there are many correlations with activity and it is not surprising that there should also be a correlation between gill area and activity.

A just criticism of Table I could be that in many cases only one or two determinations were made and with the possibility of such a large potential experi-

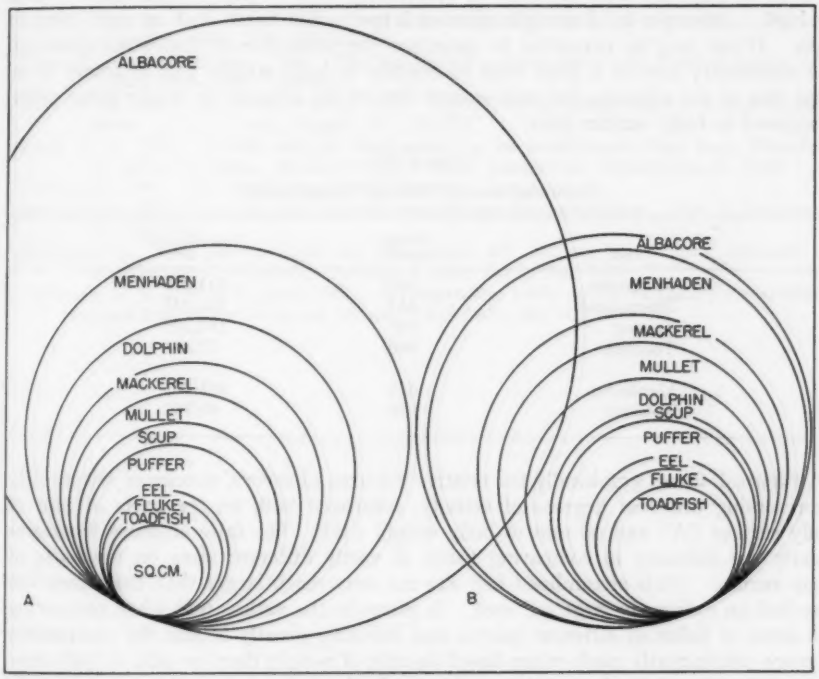

FIGURE 1. Relative gill areas of marine fishes. A. Per sq. cm. of body surface area. B. Per gram of body weight.

mental error there might arise a question of accuracy. However, if we consider only the seventeen species for which four or more determinations were made, there still remains a definite correlation with activity. This is shown in Table II where rank in order of number of lamellae per mm. of filament, gill area per gram of body weight and gill area per square centimeter of body surface are given. The order shifts a little in the three columns but the principle still holds: active fishes in general have more lamellae per mm. of filament, and greater respiratory area per unit of weight or per unit of body surface.

A more serious criticism is that the fish studied are not of the same size. This is especially significant when using body surface area as a basis for comparison, for a large fish has a relatively smaller body surface area in proportion to body weight than does a smaller fish of the same species. At first glance, the respiratory area of the false albacore per square centimeter of body surface may seem out of line. Unfortunately only one determination was made so that there can be only speculation as to accuracy. However, the surface area of this fish was in line with that of other scombrids (Gray, 1953), and gill area determinations were rechecked. This is a muscular, fast swimming fish with relatively small body surface area and it is to be expected that the ratio of respiratory area to body surface area would be high. Attempts to obtain gill areas of larger ocean fishes such as tuna were in vain. If one may be permitted to speculate, the prediction is that when obtained, the respiratory area of a large tuna in relation to body weight will be found to be near that of the albacore, but will exceed that of the albacore by many times when compared to body surface area.

TABLE III

Respiratory area of fishes of the same weight

Fish	Weight (gms.)	Total gill area (sq. mm.)
Menhaden	540	821,079
Sheepshead	544	254,237
Tautog	547	198,201
Toadfish	560	72,871
Menhaden	620	858,322
Toadfish	620	86,867

Figure 1 shows graphically the relative gill area of several species of teleost fish, representing different degrees of activity, compared both on the basis of unit of body surface (A) and on unit of body weight (B). The false albacore illustrates clearly the difficulty in comparing fishes of vastly different sizes on the basis of body surface. This streamlined fish was not only much larger than the others but also had an extremely large gill area. It seems to the author that when comparing gill areas of fishes of different species and differing greatly in size the comparison is more satisfactorily made when based on unit of weight than on unit of body surface area. Certainly the weight can be obtained much more easily and accurately than can body surface area.

Table III shows that when fishes of approximately the same weight are compared there is a definite species difference in respiratory area. It is seen here that the respiratory area of the active menhaden is roughly ten times that of the sluggish toadfish of the same weight, and that the moderately active sheepshead and tautog occupy an intermediate position.

SUMMARY

1. The gill areas of 31 species of marine teleost fishes have been compared.

2. Active, fast swimming, schooling, streamlined fishes (such as menhaden, mackerel, bluefish) have relatively much greater gill areas than do sluggish, benthic

species (toadfish, goosefish, flounders). Fishes of moderate activity (scup, sheepshead, sea bass, sea robin, puffer) are also intermediate in gill area.

3. Species differences in gill area exist whether comparison is based on unit of body surface area or on unit of body weight.

4. In general benthic fishes have fewer gill lamellae spaced farther apart than do fast swimmers.

LITERATURE CITED

GRAY, I. E., 1953. The relation of body weight to body surface area in marine fishes. *Biol. Bull.*, **105**: 285–288.

GRAY, I. E., AND F. G. HALL, 1930. Blood sugar and activity in fishes with notes on the action of insulin. *Biol. Bull.*, **58**: 217–224.

HALL, F. G., AND I. E. GRAY, 1929. The hemoglobin concentration of the blood of marine fishes. *J. Biol. Chem.*, **81**: 589–594.

HALL, F. G., AND F. H. MCCUTCHEON, 1938. The affinity of hemoglobin for oxygen in marine fishes. *J. Cell. Comp. Physiol.*, **11**: 205–212.

PRICE, J. W., 1931. Growth and gill development in the small-mouth black bass, *Micropterus dolomieu*, Lacépède. *Franz Theodore Stone Laboratory, Contribution*, **4**: 1–46.

PUTTER, A., 1909. Die Ernährung der Wassertiere. Jena.

RIESS, J. A., 1881. Der Bau der Kiemenblatter bei den Knochenfischen. *Arch. f. Naturgesch.*, **47**: 518–550.

SCHÖTTLE, E., 1931. Morphologie und Physiologie der Atmung bei wasser-, schlamm- und landlebenden Gobiiformes. *Zeitschr. f. wiss. Zool.*, **140**: 1–115.

VERNBERG, F. J., AND I. E. GRAY, 1953. A comparative study of the respiratory metabolism of excised brain tissue of marine teleosts. *Biol Bull.*, **104**: 445–449.

NITROGEN METABOLISM OF THE SLIME MOLD DICTYOSTELIUM DISCOIDEUM DURING GROWTH AND MORPHOGENESIS [1]

JAMES H. GREGG, ALICE L. HACKNEY [2] AND JEROME O. KRIVANEK

Department of Biology, University of Florida, Gainesville, Florida

Biologists concerned with the biochemistry of developing organisms would profit from analyzing a system in which the processes of growth occur independently from those of morphogenesis. The desirability of such a system has been emphasized by Needham (1942). The slime mold, *Dictyostelium discoideum* Raper, an organism exhibiting this phenomenon in nature, has been described by numerous authors, among them being Raper (1941) and Bonner (1944, 1947). Hirschberg and Rusch (1950, 1951) have indicated the suitability of the slime mold for studies of the biochemistry of development.

In view of current findings, however, there would seem to be no further justification for assuming the morphogenetic phase of development of *D. discoideum* as being devoid of growth processes (Wilson, 1952, 1953). In consequence it may be necessary to exercise caution before associating biochemical and other data with supposed morphogenetic phenomena. However, the mitoses and meioses occur only during particular periods of morphogenesis and not at all under certain conditions (Bonner and Frascella, 1952). Therefore, Bonner and Frascella (1952) suggest that morphogenetic movements are not dependent on mitoses occurring simultaneously.

This investigation is concerned with the nitrogen metabolism occurring in the slime mold and fragments of the slime mold during growth and morphogenesis. It is also the concern of this paper to associate these biochemical changes, insofar as possible, with morphological changes which occur during development of the slime mold.

Methods for Determining μg. N/μg Dry Wt.[3]

The slime molds [4] were cultured according to the method of Bonner (1947). As individual slime mold pseudoplasmodia vary tremendously in size (at least 0.3–1.5 μg. total N) (Gregg, 1950) it was necessary to study nitrogen metabolism changes on the basis of dry weight. In order to weigh samples of slime mold tissue it was necessary to utilize a quartz helical balance having a range of from 1.0–1000.0 μg. The slime molds of various stages and fragments of pseudoplasmodia (Fig. 1) were placed upon tared bits of washed and dried cigarette paper (area approx. 12 sq. mm.). The slime mold tissues were dried in a vacuum desiccator

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[2] Present address: School of Medicine, Vanderbilt University, Nashville, Tennessee.

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[4] The cultures of *D. discoideum* were kindly provided by Dr. John Bonner, Dept. of Biology, Princeton University, Princeton, N. J.

TABLE I

Ranges of dry weight and nitrogen determined by analyses of whole and fragments of slime molds during the growth and morphogenetic stages

Type of determination	Vegetative amoebae	Migrating pseudoplasmodia	Mature sorocarps	Spores	Stalks
μg. dry weight	56.2–179.0	70.4–203.0	35.6–127.0	40.5–146.0	24.2–47.6
μg. nitrogen	5.35–18.0	4.40–20.8	3.03–11.6	3.38–14.6	0.38–2.96

at room temperature or in a drying oven at 60° C. for approximately twelve hours. The samples were then weighed on the quartz helical balance (Table I). The weighed samples of slime mold tissues were then analyzed for total N (TN). The magnitude of the dry weight and total nitrogen of the samples is shown in Table I.

The nitrogen determinations were made by a modification of the method of Bruel *et al.* (1946). The results were expressed as μg N/μg dry wt. Controls were conducted during the experiments by analyzing cigarette paper for the presence of traces of nitrogen.

GROWTH PHASE

Vegetative amoebae: Figure 1

The vegetative amoebae were prepared for analysis by the following steps:

1. Harvested from four Petri dishes by rubbing the agar surfaces with a glass rod in the presence of approximately 10 ml. of distilled H_2O. Amoebae and H_2O were filtered through a small bit of cotton to remove agar particles.

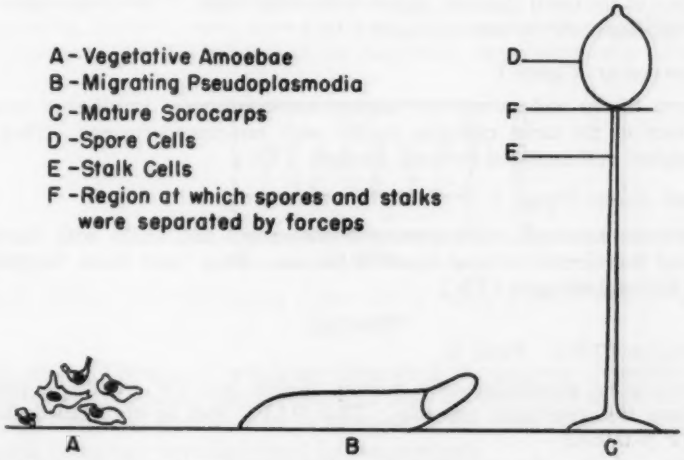


FIGURE 1. Diagram of the growth and morphogenetic stages of the slime mold, *D. discoideum*, used in the analyses.

2. Amoebae centrifuged 5–10 minutes in a 15-ml. centrifuge tube at approximately 800 g.

3. Supernatant removed and discarded. The amoebae and *E. coli* at bottom of tube placed on surface of 10 ml. of 0.95 *M* sucrose solution in a 15-ml. centrifuge tube. Material centrifuged 5 minutes at approximately 500 g.

4. Supernatant removed and discarded. Amoebae placed in fresh tube of 0.95 *M* sucrose and centrifuged 5 minutes at approximately 500 g.

5. Supernatant removed and discarded. Amoebae suspended in distilled H_2O and recentrifuged 5 minutes at approximately 800 g.

6. Supernatant discarded and slime molds transferred to tared cigarette papers for drying, weighing and nitrogen analyses. The transfer was made with a thin-walled Pyrex glass pipette approximately 0.5 mm. I.D. at the tip.

Centrifugation of the slime mold amoebae and *E. coli* in 0.95 *M* sucrose tends to decrease the number of bacteria which are thrown to the bottom of the centrifuge tube. Upon microscopic examination of such preparations, very few bacteria were seen. It is believed that the relatively low numbers of bacteria present eliminate the possibility of significant interference by extraneous nitrogen in the analyses of the vegetative amoebae.

Vegetative amoebae subjected to the 0.95 *M* sucrose washing treatment, as described in steps 1–5, when placed on an agar surface aggregated and produced normal-appearing mature sorocarps.

Morphogenetic Phase

Migrating pseudoplasmodia: Figure 1

Between ten and twenty migrating pseudoplasmodia were transferred from the agar surface to the tared cigarette papers with a hair loop. The tissues were dried, weighed and analyzed for total nitrogen (TN).

Mature sorocarps: Figure 1

Between fifteen and twenty-five mature sorocarps were transferred from the agar surface to the tared cigarette papers with fine-tipped forceps. They were dried, weighed and analyzed for total nitrogen (TN).

Spores and stalks: Figure 1

The mature sorocarps were separated into spores and stalks with fine-tipped forceps and transferred to tared cigarette papers. They were dried, weighed and analyzed for total nitrogen (TN).

Results

Total nitrogen (TN). Table II

The migrating pseudoplasmodia contain slightly less TN relative to their dry weight than the vegetative amoebae. This 9.13% loss is not statistically significant ($P > 0.05$).[5]

However, during the transition from the migrating pseudoplasmodia to the

[5] P values in this investigation were calculated by Student's t-test.

mature sorocarps, the TN/dry wt. ratio decreases 14.6% relative to that of the migrating pseudoplasmodia. Thus the mature sorocarps have been shown to contain significantly less TN/dry wt. than the migrating pseudoplasmodia ($P < 0.01$).

Analyses of the spores and stalks separately have shown that the observed loss of TN/dry wt. from the mature sorocarp did not come from the spores during

TABLE II

Analyses of nitrogen components of the vegetative and morphogenetic stages expressed as μg N/dry weight. These ratios represent the actual values with their standard deviations × 10^3. The dry weight values necessary in determining the final values of the various components other than total nitrogen were calculated from the total nitrogen/dry weight data. The numerals in parentheses refer to the number of experiments performed.

Nitrogen component	Vegetative amoebae	Migrating pseudoplasmodia	Mature sorocarps	Spores	Stalks
TN	10.30 ±0.691 (8)	9.36±1.52 (14)	7.99±0.650 (14)	9.59±2.94 (11)	4.73 ±1.55 (11)
TEN	5.70 ±2.47 (7)	6.81±0.730 (4)	4.49±0.976 (4)	6.20±2.80 (5	3.30 ±1.33 (4)
TEPN	4.75 ±2.61 (7)	5.66±0.260 (4)	2.81±0.957 (4)	2.63±0.656 (5)	0.987±0.333 (4)
TNPN	0.999±0.315 (7)	1.36±0.575 (4)	2.31±0.784 (4)	1.86±0.469 (5)	1.71 ±0.212 (4)
TUN	5.22 ±2.54 (7)	3.77±0.409 (4)	3.61±0.735 (4)	4.83±1.04 (5)	2.00 ±0.684 (4)
TUN+TEPN	9.97 ±1.46 (7)	9.43±0.641 (4)	6.42±0.509 (4)	7.46±1.06 (5)	2.98 ±0.776 (4)

TN = Total nitrogen
TEN = Total extractable nitrogen
TEPN = Total extractable protein nitrogen
TNPN = Total non-protein nitrogen
TUN = Total un-extractable nitrogen
TUN + TEPN = Total un-extractable nitrogen + total extractable protein nitrogen

culmination, since actually a slight increase (2.46%) occurred in the TN/dry wt. ratio of the spores, relative to that of the migrating pseudoplasmodia. This difference was not statistically significant ($P > 0.7$).

The stalks, however, were shown to have lost a statistically significant amount of TN/dry wt. (49.5%) relative to the migrating pseudoplasmodia ($P < 0.001$).

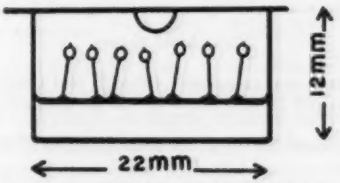


FIGURE 2. Diffusion cell with hanging drop of 0.01 N H_2SO_4 to absorb NH_3 liberated by the slime molds.

The loss of TN/dry wt. which was detected in the analyses of mature sorocarps relative to the migrating pseudoplasmodia may be attributed to an excretion of ammonia. The fact that ammonia is excreted by the slime mold *D. discoideum* in detectable quantities was determined by two methods.

The first method consisted in placing approximately twenty-four migrating pseudoplasmodia on non-nutrient or nutrient agar in a small Conway type vessel

TABLE III

Nitrogen (NH_3) excreted by D. discoideum *during the transition from migrating pseudoplasmodia to mature sorocarps determined by titration*

Mean no. of slime molds per diffusion cell	μg. N	μg. N—control	No. of experiments performed
24	—	0.545 ± 0.0153	3
0 (control)	0.009	—	1

(Fig. 2). A drop (11.8 μl.) of 0.01 N H_2SO_4 was placed on a paraffined glass slide, inverted and sealed to the vessel by means of a paraffin-vaseline mixture or Lubriseal stopcock grease. The slime molds were allowed to culminate completely. The drop of 0.01 N H_2SO_4 was then titrated to a methyl red end-point with a micro burette containing 0.01 *M* NaOH (Gregg, 1950) (Table III).

The second method was conducted similarly to the first with the exception that approximately 2.0 μl. of Nessler's reagent was added to the acid drop rather than titrating it. Nessler's reagent gave a strong positive reaction, demonstrating the presence of ammonia in the acid drop. Controls involving Nessler's reagent gave very weak positive results relative to the experimentals. These experiments have shown beyond doubt that ammonia is lost from the organism during certain of its developmental processes.

With a view to detecting possible changes in nitrogenous components which might be masked if total nitrogen analyses alone were considered, the various stages and fragments of the slime molds were fractionated in the manner described in Table IV. The fractions were designated as total extractable nitrogen, total extractable protein nitrogen, total non-protein nitrogen, total un-extractable nitrogen and total un-extractable nitrogen + total extractable protein nitrogen.

TABLE IV

Scheme designating source of various nitrogen components of slime mold tissues analyzed by micro-Kjeldahl procedure. (Modified from Gregg and Ballantine, 1946)

Slime mold tissues

↓

Homogenize in extractant composed of 0.65% NaCl in 0.01 *M* phosphate buffer at pH 7.2 and dilute to 150 μl. with extractant

↓

Remove 20–30 μl. for total nitrogen (TN)

↓

Centrifuge remainder 15 minutes at 1000 g in Misco Air turbine

Particulate matter analyzed for total unextractable nitrogen (TUN)	Remove 30 μl. of supernatant for analysis of total extractable nitrogen (TEN)	Remove 80 μl. of supernatant. Deproteinize with 15 μl. of 0.67 H_2SO_4 followed by 8 μl. of 10% Na_2WO_4. Centrifuge 15 minutes at 1000 g in Misco air turbine. Analyze precipitate for total extractable protein nitrogen (TEPN). Analyze entire supernatant for total non-protein nitrogen (TNPN).

The slime molds were harvested from the agar surfaces by the techniques described previously (Steps 1–5) with the exception that the preparations were homogenized immediately on the ground glass surface of a micro-homogenizer (Fig. 3) rather than being subjected to a weighing procedure on the quartz helical balance. The magnitude of the nitrogen present in each of the five components is listed in Table V.

Total extractable nitrogen (TEN). Table II

The 12.0% increase in TEN/dry wt. of the migrating pseudoplasmodia was not shown to be statistically significant relative to that of the vegetative amoebae ($P > 0.3$). The TEN/dry wt. content of the mature sorocarps exhibited a 34.1% decrease from that of the migrating pseudoplasmodia. This difference is statistically significant ($P < 0.01$).

From the examination of the TEN/dry wt. values of the spores and stalks it may be seen that a 51.5% loss from the stalks constitutes the major part of the decrease noted in the mature sorocarps. This difference is statistically significant

TABLE V

Ranges of nitrogen in various components determined by analyses of whole and fragments of the slime molds during the growth and morphogenetic stages

Nitrogen component analyzed	Vegetative amoebae	Migrating pseudoplasmodia	Mature sorocarps	Spores	Stalks
μg. TN	4.29–18.1	2.21–10.0	0.85–3.33	1.02–3.37	0.34–0.82
μg. TEN	1.43–19.8	4.13–12.1	2.28–4.69	0.89–5.87	0.27–0.75
μg. TEPN	2.59–47.1	11.0–20.9	2.89–6.49	0.89–3.26	0.27–0.75
μg. TNPN	1.45–5.50	2.0–6.33	1.60–6.36	0.57–2.46	0.56–1.11
μg. TUN	17.7–37.7	12.3–25.0	4.21–21.6	3.62–10.9	1.23–1.63

($P < 0.01$) while the 8.96% difference in TEN/dry wt. between the spores and migrating pseudoplasmodia was not statistically significant ($P > 0.6$).

Total extractable protein nitrogen (TEPN). Table II

The 11.9% increase in TEPN/dry wt. which the migrating pseudoplasmodia exhibit relative to that of the vegetative amoebae is not statistically significant ($P > 0.4$). The mature sorocarps, however, show a significant decrease of 50.4% of their TEPN/dry wt. when compared to that of the migrating pseudoplasmodia ($P < 0.01$). The decrease occurs from both the spores and the stalks. Values of other nitrogen components of the spores, specifically, TN/dry wt., TEN/dry wt., and TUN/dry wt., suggested that little nitrogen utilization occurs in that region. The 53.5% decrease of the spore TEPN/dry wt. from that of the migrating pseudoplasmodia is statistically significant ($P < 0.001$) and is indicative of protein metabolism within the spores which has been masked in previous analyses by the presence of other nitrogenous components, namely TNPN/dry wt. and TUN/dry wt. The stalks show a decrease of TEPN/dry wt. amounting to 82.5% of the initial amount present in the migrating pseudoplasmodia. This difference is statistically significant ($P < 0.001$).

Total extractable non-protein nitrogen (*TNPN*). Table II

The value of the TNPN/dry wt. of the migrating pseudoplasmodia, although 36.1% greater than that of the vegetative amoebae, is not significantly different ($P > 0.1$). The mature sorocarps show a 69.9% greater TNPN/dry wt. content than the migrating pseudoplasmodia. Statistical analysis, however, fails to confirm what appears to be a significant difference ($P > 0.05$). The spores and stalks show increases relative to the migrating pseudoplasmodia of 36.8% and 25.7%, respectively. Neither of these increases is significantly different from the migrating pseudoplasmodia ($P > 0.1$ and $P > 0.2$). It is possible to compute the entire amount of non-protein nitrogen produced by making the assumption that the losses observed between certain of the TN/dry wt. values of the various stages resulted from an excretion of non-protein nitrogen. These values, in addition to the re-

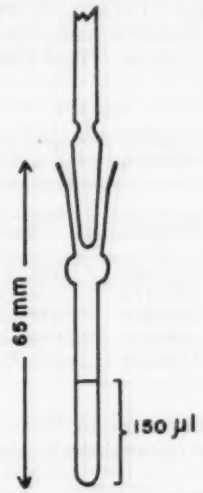


FIGURE 3. Micro-homogenizer constructed from a 5/20 Pyrex interconnecting joint.

tained non-protein nitrogen values, comprise the entire quantity of non-protein nitrogen produced. Figure 4 demonstrates that the observed increase of the entire TNPN/dry wt. during development may be attributed to a quantitatively similar utilization of the TEPN + TUN/dry wt. component. The greatest discrepancy is found at the migrating stage since the level of TNPN/dry wt. is in excess of that which can be accounted for by a breakdown of the TEPN + TUN/dry wt. component. The excess TNPN/dry wt. can possibly be attributed to digestion of ingested *E. coli* with consequent production of non-protein nitrogen.

Total un-extractable nitrogen (*TUN*). Table II

The TUN/dry wt. content of the migrating pseudoplasmodia shows a decrease of 27.8% relative to the vegetative amoebae. This decrease is not statistically significant ($P > 0.2$). The mature sorocarps show a 4.24% decrease as compared to the migrating pseudoplasmodia. Neither this decrease nor the 28.1% increase

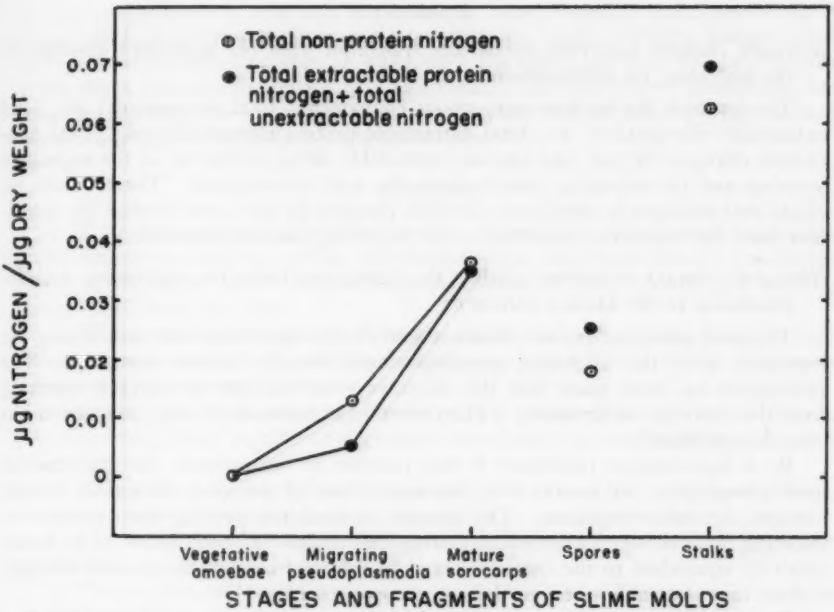


FIGURE 4. Graph relating the accumulated quantity of the TEPN + TUN/dry wt. component metabolized during development with the consequent increase in TNPN/dry wt. The accumulated TNPN/dry wt. was computed from the values of the retained TNPN/dry wt. plus that excreted from the organism as calculated from the decreases in TN/dry wt.

in the TUN/dry wt. component of the spores differs significantly from the value of the migrating pseudoplasmodia ($P > 0.7$ and $P > 0.05$). The slight but insignificant decrease in TUN/dry wt. indicated in the mature sorocarps results from the 46.9% decrease of TUN/dry wt. which occurs in the stalks. Relative to the migrating pseudoplasmodia this decrease has been shown to be statistically significant ($P < 0.01$). It appears as though this component is utilized by the stalks as well as the TEPN/dry wt. during the culmination of the slime mold.

Total extractable protein nitrogen + total un-extractable nitrogen (*TEPN + TUN*). Table II

When the sum of the two components TEPN/dry wt. and TUN/dry wt. was calculated it was found that the migrating pseudoplasmodia do not show a statistically significant decrease (5.42%) relative to the vegetative amoebae ($P > 0.5$). During culmination, however, a 31.9% decrease in the component TEPN + TUN/dry wt. occurred in comparison with the migrating pseudoplasmodia. This difference is statistically significant ($P < 0.001$). Both the spores and the stalks contributed to this decrease noted in the mature sorocarps. The decreases of the spores and stalks from the migrating pseudoplasmodia amounted to 20.7% and 68.4%, respectively. These differences are statistically significant ($P < 0.02$ and $P < 0.001$).

Discussion

Nitrogen changes occurring during the transition from the vegetative amoebae to the migrating pseudoplasmodia

The levels of the various nitrogenous components, total nitrogen/dry wt., total extractable nitrogen/dry wt., total extractable protein nitrogen/dry wt., total non-protein nitrogen/dry wt. and total un-extractable nitrogen/dry wt. of the vegetative amoebae and the migrating pseudoplasmodia were investigated. These values indicate that statistically significant nitrogen changes do not occur during the transition from the vegetative amoebae to the migrating pseudoplasmodia.

Nitrogen changes occurring during the transition from the migrating pseudoplasmodia to the mature sorocarps

The total nitrogen/dry wt. shows a statistically significant decrease during the transition from the migrating pseudoplasmodia to the mature sorocarps. The assumption has been made that this decrease reflects a loss of nitrogen resulting from the excretion of ammonia. The excretion of ammonia during this period has been demonstrated.

By a fractionation procedure it was possible to demonstrate that this loss of total nitrogen/dry wt. results from the metabolism of the total extractable protein nitrogen/dry wt. component. The increase in total non-protein nitrogen/dry wt. resulting from nitrogen metabolism during this period has been found to be quantitatively equivalent to the decrease noted in the total extractable protein nitrogen + total un-extractable nitrogen/dry wt. component (Fig. 4).

It was of interest to determine in which region of the pseudoplasmodium particular nitrogenous components were being utilized during the transition from the migrating stage to the mature sorocarp. In order that this might be accomplished, the mature sorocarps were separated into their major morphological entities, the spores and stalks. In analyzing the spores it was found that no loss of total nitrogen/dry wt. occurs. Therefore, the loss of total nitrogen/dry wt. observed in the intact mature sorocarps can be attributed to a decrease of total nitrogen/dry wt. in the stalks. The nitrogenous components responsible for the decrease of total nitrogen/dry wt. in the stalks were shown to be total extractable protein nitrogen/dry wt. and total un-extractable nitrogen/dry wt. The spores, too, show a decrease in total extractable protein nitrogen/dry wt., although of a smaller magnitude than that of the stalks. The spores do not show a loss in total nitrogen/dry wt. as simultaneous increases of other nitrogenous components are sufficient to mask the loss of total extractable protein nitrogen/dry wt.

From these data it is reasonable to suggest that both pre-spores and pre-stalks utilize proteins during the culmination process of morphogenesis. Since the pre-stalk cells are primarily responsible for raising the spore mass (Bonner, 1944; Raper and Fennell, 1952) it is not surprising that the metabolic requirements of the stalk-forming cells differ somewhat from those of the spore cells. During culmination the stalk cells build a cellulose sheath around themselves and cellulose is deposited in the walls of the spore cells (Raper and Fennell, 1952). It is suggested that the slime mold probably does not carry sufficient carbohydrate reserves both to synthesize cellulose and to utilize for purposes of obtaining energy. In this event proteins could be converted into cellulose or used for energy production.

While it is difficult to establish for which of these events protein metabolism is taking place, it is interesting to point out that the major nitrogen changes occur during the culmination process, at which time the spore and stalk cells are formed. While these data are suggestive of a relationship between protein metabolism and cellulose synthesis the hypothesis does not preclude the activity of other intrinsic mechanisms dependent upon protein breakdown.

Summary

1. Equipment and procedures incidental to determining the nitrogen metabolism of the vegetative amoebae, whole pseudoplasmodia, and fragments of pseudoplasmodia of the slime mold *Dictyostelium discoideum* during growth and morphogenesis have been described.

2. During the transition from the vegetative amoebae to the migrating pseudoplasmodia no statistically significant changes were found in any of the nitrogenous components under investigation.

3. During the transition from the migrating pseudoplasmodia to the mature sorocarps statistically significant decreases were found in certain nitrogenous components.

4. By analyzing spores and stalks separately it was possible to attribute nitrogen changes occurring in the intact mature sorocarps to particular regions.

5. The excretion of ammonia during the transition from the migrating pseudoplasmodia to the mature sorocarps was demonstrated.

6. The relationship between nitrogen metabolism and the synthesis of cellulose was discussed.

LITERATURE CITED

Bonner, J. T., 1944. A descriptive study of the development of the slime mold *Dictyostelium discoideum*. *Amer. J. Bot.*, **31**: 175–182.

Bonner, J. T., 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.*, **106**: 1–26.

Bonner, J. T., and E. B. Frascella, 1952. Mitotic activity in relation to differentiation in the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.*, **121**: 561–572.

Bruel, D., H. Holter, K. Linderstrøm-Lang and K. Rozīts, 1946. A micro method for the determination of total nitrogen (accuracy 0.005 μg. N). *C. R. Lab. Carlsberg, Ser. Chim.*, **25**: 289–323.

Gregg, J. R., and R. Ballantine, 1946. Nitrogen metabolism of *Rana pipiens* during embryonic development. *J. Exp. Zool.*, **103**: 143–168.

Gregg, J. H., 1950. Oxygen utilization in relation to growth and morphogenesis of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.*, **114**: 173–196.

Hirschberg, E., and H. P. Rusch, 1950. Effects of compounds of varied biochemical action on the aggregation of a slime mold *Dictyostelium discoideum*. *J. Cell. Comp. Physiol.*, **36**: 105–114.

Hirschberg, E., and H. P. Rusch, 1951. Effect of 2, 4-Dinitrophenol on the differentiation of the slime mold *Dictyostelium discoideum*. *J. Cell. Comp. Physiol.*, **37**: 323–336.

Needham, J., 1942. Biochemistry and morphogenesis. Cambridge University Press.

Raper, K. B., 1941. Developmental patterns in simple slime molds. *Growth* (Symposium), **5**: 41–76.

Raper, K. B., and D. I. Fennell, 1952. Stalk formation in *Dictyostelium*. *Bull. Torrey Bot. Club*, **79**: 25–51.

Wilson, C. M., 1952. Sexuality in the Acrasiales. *Proc. Nat. Acad. Sci.*, **38**: 659–662.

Wilson, C. M., 1953. Cytological study of the life cycle of *Dictyostelium*. *Amer. J. Bot.*, **40**: 714–718.

EVIDENCE FOR A SWARMING SUBSTANCE[1] WHICH STIMULATES COLONY FORMATION IN THE DEVELOPMENT OF PEDIASTRUM DUPLEX MEYEN[2]

JOHN G. MONER

Department of Biology, Princeton University, Princeton, New Jersey[3]

The life cycle of the colonial, green alga *Pediastrum* has been known since the studies of Braun (1855), Askenasy (1888), Smith (1916) and Harper (1913, 1916, 1918a, 1918b). The colonies or coenobia of this organism are commonly found in fresh water and are disk-shaped in form. The cells of the colony are arranged in a single layer with the marginal cells usually differing from those of the interior by possessing one or two prongs or processes produced radially outward. The number of cells in a colony varies but in general, can be described by the simple formulation 2^N, the common range being eight to sixty-four. Asexual reproduction involves the formation of biflagellate zoospores which arise through successive nuclear divisions, followed by progressive cleavage of the cell chloroplast. After the zoospores have been formed they are discharged through a crescent-shaped slit in the mother cell wall and are enclosed in a thin, transparent sac or vesicle. At first, they form an irregularly shaped mass, but after three to four minutes of active movement, they slow down forming a flat plate of cells. Complete cessation of movement then follows and prong formation begins and is completed in a matter of minutes.

Although details of the life cycle have long been known, little work has been done concerning the factors affecting the production of zoospores and their release to form new colonies. The purpose of the study presented in this paper was to examine the characteristics of growth and reproduction in *Pediastrum* when grown in culture and to investigate the role of certain factors which influence these processes. The results will be concerned with such aspects as the time at which colony formation begins and how its initiation is affected by pH and aged medium. Evidence will be presented for the occurrence of a substance or group of substances in aged medium which can alter the time at which colony production begins, the number of colonies produced and the size of these colonies in terms of cell number.

Materials and Methods

The species of *Pediastrum* used in this study was *P. duplex* Meyen (from Malham) and was provided by E. G. Pringsheim of the Cambridge University Botany School in unialgal, non-bacteria-free culture. It was grown in this form

[1] The word "substance" is used to denote one or more substances active in stimulating colony formation in *Pediastrum duplex* Meyen.

[2] This paper represents a part of a dissertation in Biology presented to the faculty of Princeton University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Present address: Tropical Research Medical Laboratory, A.P.O. 851 C/O Postmaster, New York, New York.

on mineral medium No. 8 according to Chu (1942) with 10% soil extract added. All of the cultures used in this section were prepared under sterile conditions and grown in Ehrlenmeyer flasks.

For most of the experiments the culture vessels were placed in a trough with tap water circulating through it to maintain a relatively constant temperature. The lighting was provided by two 15-watt daylight General Electric "fluorescent" bulbs set parallel 26 cm. apart in a wooden housing and elevated 25 cm. above the culture-containing trough. In one experiment only (Section A), the culture was not grown in a water-circulated trough but was simply placed on a wooden platform and allowed to grow at room temperature (24–28° C.). This culture was illuminated by a 100-watt tungsten filament bulb housed in a desk-type lamp and placed 40 cm. in front of the culture. In all cases the lighting was continuous throughout the course of the experiment. It should be noted that temperature was not continuously recorded by any mechanical device but that periodic measurements were made daily using a standard mercury thermometer. When the cultures were grown in the water-circulated trough the temperature of the circulating water was determined; when a culture was grown in the open air the temperature of a flask of water placed next to the culture flask was determined. The constancy of the light source (*i.e.*, intensity and wave-length) was also not strictly controlled. It was felt by the author that the aim of this investigation did not necessitate the use of rigid controls of temperature and light since in every experiment both control and experimental cultures were grown under identical conditions. Any investigation of the effects of temperature and light on the phenomena reported in this paper would, of course, require strict control of these two factors and it is hoped that a study of this nature will be possible during the course of future work.

For counting the number of colonies in a culture, two counting chambers were used: The Levy hemacytometer and the Sedgwick-Rafter counting chamber. For determining colony number with the Levy hemacytometer the method for counting leucocytes was used according to the following formulation:

$$\text{Colonies/cc.} = \frac{\text{Colonies counted} \times \text{dilution} \times 10^4}{\text{No. of 1 sq. mm. areas counted}}$$

To determine colony number with the Sedgwick-Rafter counting chamber the number of colonies in one pathway was counted using a 10 × objective and 20 × ocular and the following formulation used:

$$\text{Colonies/cc.} = 17 \times \text{colonies in one pathway} \times \text{dilution} \times 2.$$

The colonies were always fixed in 2–3% formalin before counting. In all experiments but one the Sedgwick-Rafter chamber was used to determine colony number.

To determine cleavage number the colonies were fixed in formalin-aceto-alcohol and stained with Harris' hematoxylin according to the method of Johansen (1940). A drop of suspension containing stained colonies was then placed under a cover slip, sealed with paraffin and the number of cells showing cleavage figures was recorded for 150 colonies counted. To make these counts the 95 × oil immersion objective and 10 × ocular were used. pH measurements were made with a Cambridge pH-Meter Laboratory Model-L.

Results

A. Quantitative relations of growth and colony production

At the outset of this work on *Pediastrum* it was desirable to obtain large numbers of swarming colonies in a relatively short time. To accomplish this, large inocula of 5 cc. for every 50 cc. of fresh medium were used in preparing cultures. When this was done it was noticed that swarming occurred in great numbers at a specific time after inoculation and that it seemed to last for only a few days. The time that swarming or colony production began could be predicted with reasonable accuracy in succeeding cultures inoculated in the same manner from the same stock. Virtually no swarming was found in the cultures prior to this swarming period and after it was completed.

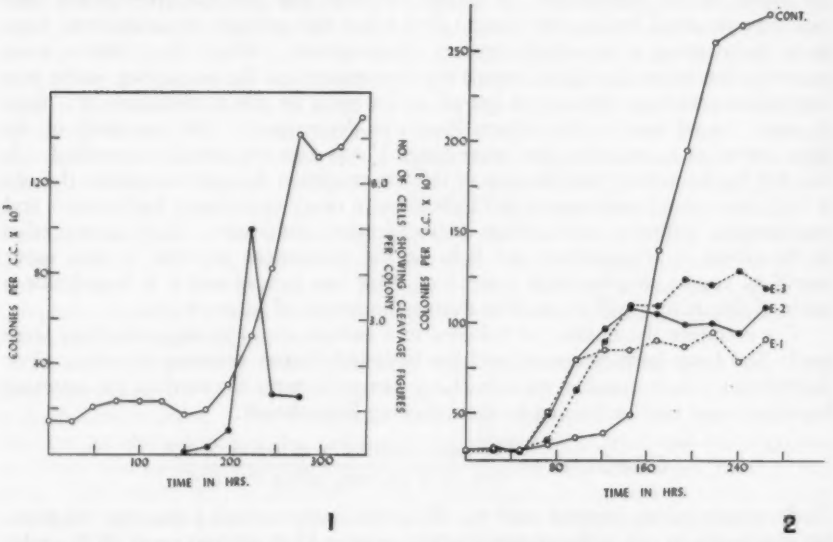


Figure 1. Graph showing colony production and cleavage activity curves. Open circles represent colony production and black dots represent cleavage activity.

Figure 2. Graph showing colony production in fresh or control medium and in aged medium after 0 (E-1), 1 (E-2) and 2 (E-3) days' growth in fresh or control medium.

In order to obtain a quantitative description of colony formation as a function of time the following experiment was performed: a culture was started consisting of 50 cc. of fresh medium inoculated with a 5-cc. suspension of 99-day old stock culture and grown in a 125-cc. flask. The lighting was provided by a 100-watt tungsten filament bulb and the experiment run at room temperature (24–28° C.; see Materials and Methods). One-cc. aliquots were removed daily from the culture, fixed and colony number determined using a Levy hemacytometer chamber. In the results in Figure 1 it is seen that no colony production occurs in the culture up to 173 hours but that a sudden burst begins at 197 hours and lasts for 78 hours at

which time it begins to level off. The final number of colonies is approximately 8 times the value of the starting colonies which is what would be expected if the original mean colony size was 8 cells (see Discussion). Cleavage counts were made to see whether cleavage activity coincided with the burst phase of the curve. In the graph of Figure 1 this activity is expressed as the number of cells showing cleavage figures per colony and it can be seen that there is a correlation between colony formation and cleavage activity. It should be noted that although no swarming takes place in the culture up to 173 hours, considerable growth, in terms of colony size, occurs during this period.

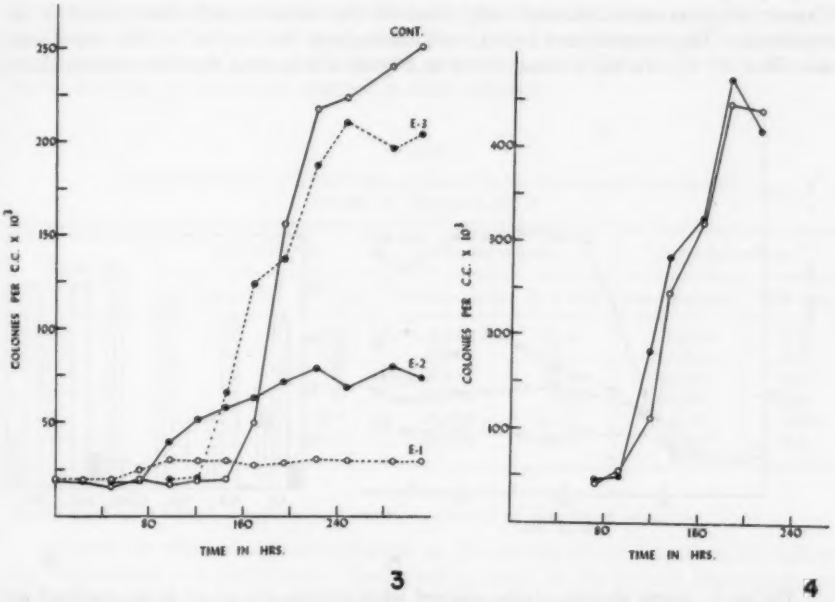


FIGURE 3. Graph showing colony production in fresh or control medium and in aged medium after 0 (E-1), 2 (E-2) and 4 (E-3) days' growth in fresh or control medium.

FIGURE 4. Graph showing results obtained when colonies are placed in aged medium one day prior to the time they will swarm in fresh or control medium. Open circles represent colony production in the control and black dots represent colony production in the experimental.

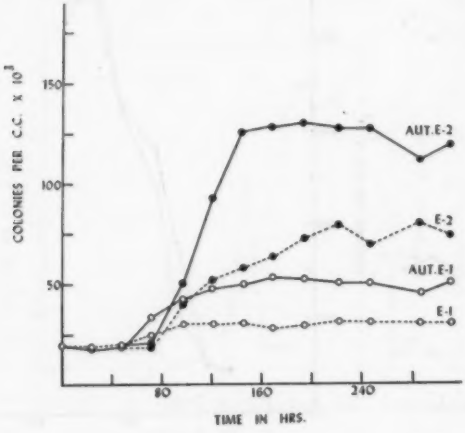
B. Evidence for a substance in the aged medium affecting colony reproduction

The curious nature of the swarming curve suggested the possibility of some change occurring in the medium which might initiate swarming or colony formation. Experiments were performed to test how colony production was affected by moderately aged medium, in which swarming had occurred. In the first experiment 150 cc. of fresh medium were inoculated with 15 cc. of a 53-day old stock culture and placed in a 500-cc. flask to serve as standard culture. Aged medium was obtained from a 19-day old culture from which the colonies had been removed

by centrifugation. Using the standard culture and aged medium the following cultures were prepared:

Control: 30 cc. of standard culture. Prepared at zero time.
Experimental No. 1: Colonies from 30 cc. of standard culture, fresh medium removed and replaced by 30 cc. of aged medium. Prepared at zero time.
Experimental No. 2: Colonies from 30 cc. of standard culture after one day's growth, fresh medium removed and replaced by 30 cc. aged medium.
Experimental No. 3: Colonies from 30 cc. of standard culture after two days' growth, fresh medium removed and replaced by 30 cc. aged medium.

One-cc. aliquots were removed daily from all the cultures and colony number determined. The temperature maintained throughout the course of the experiment was $20 \pm 2°$ C. In the results shown in Figure 2 it is seen that the control shows



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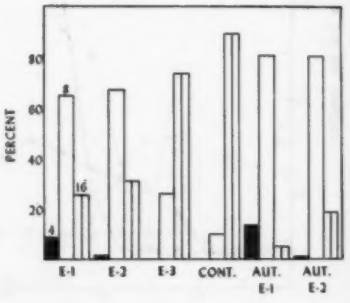

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FIGURE 5. Graph showing results obtained when colonies are grown in unautoclaved and autoclaved aged medium after 0 (E-1) and 2 (E-2) days' growth in fresh or control medium.
FIGURE 6. Graph showing the final percentage distribution of four-, eight- and sixteen-celled colonies produced in the control and the unautoclaved and autoclaved aged medium cultures of Figures 3 and 5.

the familiar swarming burst after 144 hours but that all three experimentals show a substantial increase in colony number after 73 hours. The longer the colonies grow in fresh medium, the greater the level of colony production reached after they are transferred to aged medium. None of the experimental cultures produce as many new colonies as the control.

In a repeat experiment similar to the preceding one colonies were allowed to grow in fresh or control medium for 0 (Exp. No. 1), 2 (Exp. No. 2) and 4 (Exp. No. 3) days before being transferred to aged medium. The standard culture was prepared from a 58-day old stock culture and the aged medium was obtained from

a 15-day old culture. In the results shown in Figure 3 it is apparent that all three experimental cultures show colony production before the control does. Once again, the longer the colonies grow in fresh or control medium the greater the number of new colonies they produce when transferred to aged medium. The colonies grown for 4 days in fresh or control medium prior to transfer to aged medium show a final level of colony production very close to that of the control.

Figure 4 shows the results obtained when colonies, one day prior to the time they will normally begin swarming, are placed in 8-day old aged medium. In this experiment the colonies had been growing three days in fresh medium when they were placed in aged medium. The standard culture was prepared using 87-day old stock culture and the temperature maintained throughout the course of the experiment was $19 \pm 2°$ C. It is seen that although both cultures begin swarming at the same time the experimental shows a greater burst. Essentially the same final level of colony production is reached in both cultures.

TABLE I

Final distribution of colony types produced in the control and experimental cultures of Figures 3 and 5.

Culture	4-celled colonies		8-celled colonies		16-celled colonies	
	New colonies/cc.	% of total	New colonies/cc.	% of total	New colonies/cc.	% of total
E-1	1,112	8.52	8,588	65.8	3,356	25.7
E-2	756	1.52	33,568	67.6	15,331	30.8
E-3	0	0	50,837	26.0	144,983	74.0
Control	0	0	20,624	9.95	186,497	90.0
Aut. E-1	4,427	13.9	25,826	81.2	1,571	4.94
Aut. E-2	1,163	1.17	80,294	80.5	18,403	18.4

C. The effect of high temperature on the activity of the aged medium

To test the effect of high temperature on the activity of aged medium the following experiment was performed: Aged medium used in the second experiment reported in section B was autoclaved for 30 minutes under 15 lbs./in.^{2} pressure at 121° C. Colonies were placed in this autoclaved aged medium after 0 and 2 days growth in fresh medium and the cultures were run simultaneously with Experimentals No. 1 and No. 2 of the second experiment reported in section B, both of which contained unautoclaved, aged medium. From the results shown in Figure 5 it is evident that the two autoclaved experimentals show colony production at the same time as the unautoclaved counterparts. However, the colonies placed in autoclaved aged medium after 0 days growth in fresh medium produce 3 times as many new colonies as the unautoclaved partner, while those placed in autoclaved aged medium after two days' growth in fresh medium produce almost twice as many new colonies as the unautoclaved partner.

Table I and Figure 6 show the per cent distribution of 4-, 8- and 16-celled colonies produced by the control and three experimental cultures of the second experiment reported in section B and the two autoclaved aged medium experi-

mentals of this section. The results show that the longer the colonies are kept in the control medium before transfer to aged medium, the greater the tendency to produce colonies with larger cell number.

D. The role of pH of the medium

Two experiments were performed to determine whether the effect of aged medium in causing premature colony formation was due to pH change in the medium. Since the medium is poorly buffered it was thought that pH change

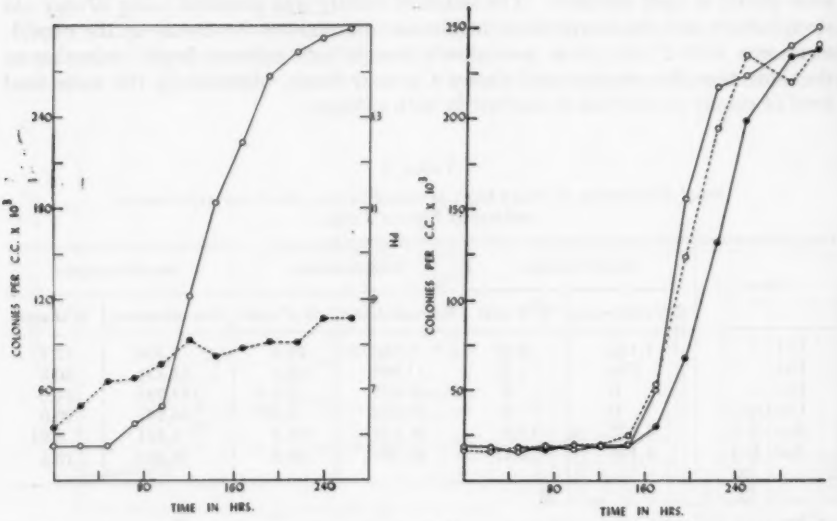


FIGURE 7. Graph showing curves of colony production and pH change of the medium during colony production. Open circles represent colony production and black dots represent pH change.

FIGURE 8. Graph showing results obtained when colonies are grown in pH-regulated medium (7.31) after 0 (E-1) and 2 (E-2) days' growth in fresh or control medium. Open circles connected by continuous line represent colony production in the control; open circles connected by dotted line represent colony production in pH-regulated E-1 and black dots represent colony production in pH-regulated E-2.

might be an influencing factor. A culture was started consisting of 200 cc. of fresh medium inoculated with 20 cc. of 56-day old stock culture and grown in a 500-cc. flask. Two (5-cc.) aliquots were removed daily and pH and colony number determinations made. The temperature maintained throughout the course of the experiment was 19 ± 2° C. Figure 7 shows the results obtained. It is clear that change of pH from the acid to the basic side occurs during the growth and reproduction of *Pediastrum* under these culturing conditions; pH changed from an initial 6.13 to a final 8.60 but there does not appear to be any sudden increase in pH

at the time of colony formation. Instead a gradual, continuous increase occurred both prior to and during this phase.

Since pH change did occur in the medium during the development of *Pediastrum* another experiment was performed to test whether this change was the causative influence in the premature swarming effect of aged medium. In this experiment th pH of fresh medium (6.29) was regulated with 0.1 N NaOH to that of the aged medium used in the second experiment of section B (7.31). Colonies were placed in this pH-regulated medium after 0 and 2 days' growth in fresh, unregulated medium and the culture was run simultaneously with the control and Experimental No. 1 and No. 2 of the second experiment of section B. In the results shown in Figure 8 it is seen that both pH-regulated cultures show the swarming burst at approximately the same time as the control. The colonies receiving the pH-regulated medium after two days' growth in fresh unregulated medium apparently respond somewhat later than those of the control. When the experiment was completed the pH of the control was 7.51, the 2-day aged medium experimental (Exp. No. 2) was 7.50 and the 2-day pH regulated experimental was 7.72.

Discussion

It has been shown for the first time that colonies of *Pediastrum* when grown in cultures using large inocula in fresh media exhibit two developmental phases. The first is a latent period which may last for 3–7 days, in which the colonies grow but do not reproduce. The second phase is a very active period of swarming or colony production which lasts 3–4 days during which all the cells in the initial inoculum give rise to new colonies. Cleavage activity, as would be expected, has been shown to be quite synchronous with this period. That essentially all the cells in the original colonies give rise to new colonies can be demonstrated by determining the average number of cells per colony in the inoculum and multiplying this value by the number of colonies present. In the experiment concerning colony type distribution the control showed an average colony size of 14.0 cells/colony and a colony number of 18,718/cc. in the starting culture. If all the cells gave rise to colonies then the value reached by the control after swarming should be (14.0) (18,718) or 262,052/cc. The actual value reached by the control is reasonably close: 252,450/cc.

The fact that cultures exhibit a latent period might have initially been interpreted as some inherent time lag in the ability of the colonies to reproduce during this period. However, the results show that by placing colonies in medium in which swarming has occurred colony formation can be induced prematurely. The longer the preliminary period of growth in control or fresh medium, the greater the number of colonies produced after transfer to aged medium and the more closely the resulting swarming curve resembles that of the control. Coincident with these changes is the gradual increase in colony size in terms of cell number as the preliminary period of growth in control or fresh medium is lengthened. Again, the longer this preliminary period of growth the more closely the colony type distribution resembles that of the control. It appears evident that the latent period in the normal swarming curve of *Pediastrum* is one in which the competence of the colonies to reproduce is ever increasing. This might conceivably be a function of critical size whereby colonies which are too small and immature to reproduce are

transformed, during the period of growth, into colonies of adequate size. The longer this period of growth the more abundant the number of colonies reaching reproductive maturity. The fact that colonies can reproduce during the latent period, and yet do not, indicates that the proper stimulus for reproduction is not present at this time. The evidence indeed indicates that the swarming burst is a response to some substance or substances accumulating in the medium during the growth of the colonies and that the latent period is simply an index of the time required for the substance to reach a threshold concentration. That the action of this substance is not through a pH effect has been shown by placing colonies in medium the pH of which has been regulated to that of aged medium and yet the pH-regulated medium has no stimulating effect but parallels normal control medium. The substance is not only heat-stable, but for some reason, has its activity enhanced by high temperature. This increase in activity could conceivably be caused by the removal or breakdown of inhibitory factors present in the medium, or to the production of breakdown products which can further induce colony formation.

The origin of the swarming substance cannot be determined unequivocally from these experiments. The possibility of bacterial origin exists and can only be ruled out through repeat experiments with pure cultures.

It is known, however, that certain algae do produce substances capable of influencing growth. Lefevre, Jacob and Nisbet (1952) in their studies on various fresh water algae have found evidence for the production of substances which can inhibit not only the growth of the algae producing them (autoantagonism) but the growth of other species of algae as well (heteroantagonism). Extensive work with *Pandorina* and *Scenedesmus* has revealed the production by these organisms of inhibitors which the authors have named Pandorinine and Scenedesmine, respectively. These substances, in addition to being autoantagonistic in their inhibitory power, will inhibit the growth of several other algae including various species of *Pediastrum*. It is indeed interesting that boiling the inhibitor usually lowered its inhibitory effect and, in some instances, actually caused it to give an enhancement of growth. This, in some respects, parallels the experiments presented in this paper regarding autoclaving of the swarming substance. It seems quite possible that at least some algae produce both growth inhibitory and stimulating substances as a normal part of their metabolism and that the former are more heat-labile than the latter.

If the swarming substance is produced by the *Pediastrum* colonies then it is most interesting to review the results of Smith (1916) who studied *P. Boryanum* in pure culture. Smith found that for the first two weeks in culture 64- and 32-celled colonies were predominant while a month later 8- and 16-celled colonies were most common. A reasonable explanation of these observations is now available. Since pure cultures in general are prepared with very small inocula it seems likely that Smith's cultures contained small numbers of starting colonies. If growing colonies were producing a swarming inductor it would take a considerable length of time for a small number to produce enough substance to initiate reproduction. The colonies would be expected to grow to a rather large size before a threshold concentration of the substance was reached and colonies with large cell numbers would be produced. However, as the culture grew older new colonies

could not grow as long for the substance would be present tending to induce reproductive activity and colonies of fewer cell number would be produced.

The role of such a substance in the free-living *Pediastrum* colony in nature would be most important. In an actively moving, nutrient-rich environment colonies could grow for an extended time without the substance collecting. In this manner maximum growth would take place and when reproduction finally occurred colonies of great cell number would be produced. In an inactive, stagnant environment, however, the substance would tend to build up and finally force reproduction. It is easy to see the survival value of an increase in numbers for dissemination to more favorable conditions. To speculate even further, it is not difficult to imagine that this swarming substance could, at the proper concentration, stimulate sexual reproduction and the formation of free-swimming gametes. There are some indications that this might be so. It is known, from the author's experience, that when concentrated suspensions of swarming colonies are left standing for a day or more, free-swimming gamete-like cells are often produced. Since fusion between these cells has never been observed it is not known for certain whether they are gametes. Askenasy (1888) found that he could initiate gamete production sooner in his cultures if the colonies were kept in a smaller volume of water. If colonies were living in a drying pond the substance would build up and finally force gamete production. This mechanism would have the advantage of bringing the sexes together in concentrated form, thereby facilitating the meeting of the sexes and fusion. Furthermore, the result of fertilization is a resistant polyhedron which is capable of surviving the drought and germinating again when the rains come and the pond fills up. This hypothesis concerning the role of the swarming substance in sexual reproduction should be capable of testing and experimental work on this subject is planned.

The author wishes to thank Dr. John Tyler Bonner for his fruitful guidance and encouragement during the course of this work.

Summary

1. The characteristics of growth and reproduction in *P. duplex* Meyen have been studied in non-bacteria-free cultures using large inocula of 5 cc. of stock culture for every 50 cc. of fresh medium.

2. It has been found that colonies grown under these conditions exhibit two phases of development: A latent period in which no colony production or swarming takes place but during which the colonies grow, and a period of active swarming or burst phase during which all the cells of the starting colonies give rise to new colonies.

3. It has also been shown that a heat-stable substance is present in aged medium which can, through an effect other than pH, alter the time at which colony production begins, the number of colonies produced and the size of these colonies in terms of cell numbers.

4. It is suggested that the length of the latent period of development is determined by the time required for this substance to reach a threshold concentration. The possible importance of such a substance to free-living forms of *Pediastrum* in nature is also discussed.

LITERATURE CITED

ASKENASY, E., 1888. Ueber die Entwicklung von *Pediastrum*. *Ber. Deutsch Bot. Ges.*, **6**: 127–138.

BRAUN, A., 1855. Algarum unicellularium Genera nova et minus cognita. Lipsiae.

CHU, S. P., 1942. The influence of the mineral composition of the medium on the growth of planktonic algae. I. Methods and culture media. *J. Ecol.*, **30**: 284–325.

HARPER, R. A., 1913. Morphogenesis in *Pediastrum*. *Science*, II, **37**: 385.

HARPER, R. A., 1916. On the nature of types in *Pediastrum*. *Mem. N. Y. Bot. Garden*, **6**: 91–104.

HARPER, R. A., 1918a. Organization, reproduction and inheritance in *Pediastrum*. *Proc. Amer. Phil. Soc.*, **57**, No. 4.

HARPER, R. A., 1918b. The evolution of cell types and contact and pressure responses in *Pediastrum*. *Mem. Torrey Bot. Club*, **17**: 210–240.

LEFEVRE, M., H. JAKOB AND M. NISBET, 1952. Auto et heteroantagonisme chez les algues d'eau douce, in vitro et dans les collections d'eau naturelles. *Annales de la Station Centrale d'Hydrobiologie appliquee*, **4**.

JOHANSEN, D. A., 1940. Plant microtechnique. McGraw Hill Book Co., N. Y.

SMITH, G. M., 1916. Cytological studies in the Protococcales. II. Cell structure and zoospore formation in *Pediastrum Boryanum* (Turp.) Menegh. *Ann. of Botany*, **30**: 467–479.

SECRETION OF GASES AGAINST HIGH PRESSURES IN THE SWIMBLADDER OF DEEP SEA FISHES.

I. OXYGEN DISSOCIATION IN BLOOD[1]

P. F. SCHOLANDER AND L. VAN DAM

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, and Lerner Marine Laboratory, Bimini, Bahamas

By studying the composition of the swimbladder gases in relation to depth it was established that both oxygen and nitrogen are deposited into the swimbladder against a concentration gradient to make up the hydrostatic pressure. The nitrogen is deposited as a certain fraction of the oxygen varying from 2 to 15%, according to the species. Carbon dioxide and organic gases contribute only insignificantly to the pressure. The oxygen pressure commonly reaches values above 100 atmospheres, and it is likely that it can be two to three times as much, as species with swimbladders are known to occur down to a pressure of at least 266 atmospheres. The nitrogen pressure in deep sea species frequently reaches 10–15 atmospheres (Scholander and van Dam, 1953).[2] In whitefish (*Coregonus*) pure nitrogen may be deposited into the swimbladder against 6–8 atmospheres' pressure (Hüfner, 1892). Saunders (1953) has verified this finding, and has added four more species of physostome fresh water fishes which also seem to deposit nothing but nitrogen in the swimbladder.

Current Theories of Gas Secretion

Haldane (1922) in his celebrated book "Respiration" gives his views as to a possible mechanism for the gas secretion, which in its basic concepts expresses the ideas offered by later authors. He saw in the *rete mirabile* a counter current exchange mechanism (p. 214): "The arrangement reminds us of that in a regenerating furnace, where the heat carried away in the waste gases is utilized to heat the incoming air." As to the chemical release of oxygen he points out the importance of CO_2 in expelling oxygen from the hemoglobin and states (p. 215): "It seems probable, therefore, that the function of the *rete mirabile* is to enable venous blood to communicate part of its CO_2 to the arterial blood. The effect of

[1] Contribution No. 699 from the Woods Hole Oceanographic Institution. This investigation was supported by a grant from the National Science Foundation.

[2] In our 1953 paper data are given on gas samples from *Alepocephalus* and *Cottunculus*. From the excellent review of Jones and Marshall (1953) we learned that *Alepocephalus* does not have a swimbladder and this we verified on our recent material. We found also that *Cottunculus* has no swimbladder. This raises the question as to where the gas came from of which we obtained a few cubic centimeters by poking around in these fishes with our needle. The presence of the gas was obvious because the fishes were floating on the water. The composition of this gas checked well with that of the other fishes in the same catch, and hence it is believed that the fish must have swallowed some of the gas gushing out from other fishes when the trawlbag approached the surface. Obviously a fish distended by swimbladder gas cannot pollute this gas by swallowing.

this will be to raise the CO_2 pressure of the blood supplied to the gland, and so raise the oxygen pressure." Already Müller (1840) and Woodland (1911), who presented accurate anatomical drawings of the *rete*, had considered that they must be interpreted as a diffusion apparatus between the arterial and venous capillaries.

Hall (1924) found that, if the gas gland in yellow perch (*Perca flavescens*) was stimulated to activity by removing gas from the swimbladder or by increasing the hydrostatic pressure, a dialysate of the active gland showed a decrease in pH from 7.1 to 6.4, as compared with a non-active gland. He assumed secretion of an acid by the glandular epithelium. This would diffuse into the capillaries of the *rete* and by releasing bound oxygen would raise the oxygen tension, permitting the oxygen to diffuse into the swimbladder. If the oxygen capacity were 9 vol. % and the solubility coefficient 3 vol. % per atmosphere, then this mechanism could raise the oxygen tension no more than 3 atmospheres. This would be ample for shallow water fish like the perch, but could not explain the secretion of 100 atmospheres or more in deep sea fishes.

Jacobs (1930) found a way out of this difficulty by elaborating somewhat further on the possible function of the *rete mirabile*. When acid is secreted by the glandular cells and diffuses into the blood, CO_2 is released, increasing the CO_2 tension. The CO_2 effect itself, or possibly the secreted acid, dissociates oxygen from the oxyhemoglobin, so that both CO_2 and oxygen tension increase. This would explain the fact that both O_2 and CO_2 tension increase when gas is generated following a puncture. The blood with elevated O_2 and CO_2 tension leaves the gland and enters the efferent (venous) *rete*, where the gases diffuse over to the afferent (arterial) side, boosting the tensions there. When this high tension blood enters the gland, new acid is added, the tensions are again stepped up, and so on, until high enough tension has been built up in the swimbladder.

The secretion of oxygen would accordingly involve two principal steps: (1) a chemical dissociation of oxygen by acid (and/or CO_2) from the oxyhemoglobin, and (2) a counter current exchange mechanism (the *rete mirabile*) which would retain the secreted gases within the swimbladder with a minimal loss to the outside, in spite of maintained circulation. These basic ideas have been accepted by almost all later workers as the likely mechanism for oxygen secretion in fishes (Powers *et al.*, 1932; Koch, 1934; Irving and Grinnell, 1939; Black, 1946; Copeland, 1952; Strittmatter, Ball and Cooper, 1952; Fänge, 1953). Von Ledebur (1937) expressed serious doubts as to the correctness of the chemical interpretation.

In the present account we shall deal with the oxygen dissociation at pressures from 0.2 to 140 atmospheres and its relation to pH and CO_2. The function of the *rete mirabile* will be dealt with in a later account.

Oxygen Dissociation in Blood from Deep Sea Fishes in Relation to pH and CO_2

The chemical or physical reaction which is in the end responsible for the oxygen deposition in deep sea fishes must be able to perform against a pressure which at least equals the partial pressure of oxygen known to occur in the fish in question. Many deep sea fishes secrete oxygen against 100 atmospheres or more, and by inference some secrete against considerably more than 200 atmospheres.

It has long been known that the blood of many fishes possesses a large Bohr effect (Krogh and Leitch, 1919, and many later authors). Root (1931) and Green and Root (1933) found that in several fishes the blood did not become completely saturated if acid or CO_2 was added at oxygen pressures up to near one atmosphere. The saturation curves were found to run virtually horizontal at pressures above 0.5 atmosphere. The *Bohr effect* is generally considered a change in the dissociation constant (K in the Hill equation) with 100% saturation obtainable at high enough pressure. The term *Root effect,* on the contrary, may be reserved for the situation where acidity completely blocks the oxygen from part of the hemoglobin.

Obviously, before we can know whether the Bohr or Root effect can be responsible for the oxygen secretion against 100–200 atmospheres' pressure, we must extend the dissociation curves to these pressures. This has been done for a series of ocean fishes inhabiting depths equivalent to pressures from 40 to as much as 260 atmospheres.

The investigation was commenced in the winter of 1952–1953 at the Lerner Marine Laboratory, Bimini, Bahamas, and was amplified by material caught on the dragger *Cap'n Bill II* during two cruises in the summer of 1953 off the New England coast.

Material

Fishes were caught at the bottom by hook and cable from 400–700 meters' depth in the Gulf Stream off Bimini between December, 1952 and February, 1953. For this purpose a gasoline-powered fishing rig was used, originally designed by Captain Eddie Wall at the Lerner Laboratory. The fishes were brought to the surface within 4–7 minutes after hooking, according to the depth. Blood was drawn immediately by hypodermic needle from the heart and transferred to a polyethylene bottle containing a little heparin. The sample, of about 20–50 cc., was stored on ice and brought to the laboratory within an hour. The blood was then put through all the necessary procedures at once.

Three species were obtained at Bimini:

			Depth of catch
3	*Epinephelus mystacinus*	Black grouper	320–400 m.
1	*Alphestes* sp.[3]	"Golden grouper"	410 m.
2	*Polyprion americanus*	Wreckfish	640 and 675 m.

The fishes weighed between 10 and 30 kg. each.

Deep sea material was likewise obtained on board the dragger *Cap'n Bill II,* operating from Woods Hole, on two cruises (June 16–23 and July 1–8, 1953). The fishes were caught by bottom trawl, mostly at depths of between 500 and 1300 meters, depending upon the species. It took up to three quarters of an hour to get the trawl to the surface from the time it left the bottom. The blood could in some cases be drawn from the heart (*Sebastes*). In other cases blood was obtained which dripped from the cut tail. It was usually necessary to pool blood from many specimens to get enough for the analyses, which were performed immediately.

[3] Called *Epinephelus,* Grouper, in Scholander and van Dam (1953).

The following species were caught and analyzed on the *Cap'n Bill:*

		Depth of catch
Antimora viola	Blue hake	1200 m.
Coryphaenoides rupestris	Round-nosed ratfish	1100 m.
Sebastes marinus[4]	Rosefish	550– 720 m.
Synaphobranchus pinnatus	Long-nosed eel	960–1100 m.
Urophycis chesteri	Long-finned hake	600–1300 m.

Optical method for determination of dissociation curves at high pressures

The problem required a study of oxygen dissociation of fish blood at different pH or CO_2 tensions carried from an oxygen pressure of 0.2 atmosphere, if possible, up to values corresponding to the greatest depth at which the species was known to occur. As a routine the highest oxygen pressure employed was that of a fully charged commercial gas cylinder, *i.e.*, 140 atmospheres.

An earlier survey (van Dam and Scholander, 1953) showed that the blood of deep sea fishes had about the same oxygen combining capacity as that of shallow fishes, *i.e.*, about 6–10 vol. %. At 100 atmospheres' pressure the blood would physically dissolve about 300–400 vol. % oxygen, and hence it would be impossible by direct gasometric methods to determine the saturation of the hemoglobin. An optical method was therefore designed.

The method must be accurate to within a few per cent saturation, and it must be possible to operate it on a rolling ship. It was necessary to equilibrate the blood sample in the photometer cuvette, which must be accurately controlled to the same temperature as that at which the bottom fishes live. The cuvette must be able without significant distortion to stand a pressure of 140 atmospheres.

As a measuring instrument a Zeiss Pulfrich Stufen Photometer [5] was employed. The construction of the high pressure cuvette will be seen in Figure 1. The glass windows were ½-inch thick and the effective depth of the blood in the optical paths was 1.6 mm., increasing 1% at 140 atmospheres. Sealing of the cuvette chamber was obtained by a Neoprene "O" ring squeezed between the windows. Entrance to the cell was by means of a number 20 hypodermic needle which pierced the "O" ring. Through this needle blood could be introduced by a number 25 needle. The cell could be cleaned by rinsing it with 0.17 *M* saline solution, which was then removed by suction. A stainless steel ball was kept in the cuvette for stirring. The cuvette was dropped into an insulated water bath occupying the position where ordinarily the cuvette holder is placed on the instrument. Water of constant temperature (4° or 10° ± 0.1° C.) circulated through the water bath. Dewing of the water bath windows was prevented by keeping them wet with soap solution. To accomplish equilibration the cuvette was only partially filled with blood (see Fig. 1), and it was rotated back and forth through an arc of 45° by means of a motor. To permit rotation the high pressure line of small gauge copper tubing was wound as a loose helix around the cuvette water bath, seven to eight turns, before it was

[4] In the paper of Scholander and van Dam (1953) the maximum depth of *Sebastes* is given as 1680 m., as recalculated from 917 fathoms given in Goode and Bean (1895, p. 261). This, however, represents a misquotation of the original figure of 179 fathoms (Townsend, 1901, p. 398).

[5] This was kindly lent to us by the Department of Biological Chemistry, Harvard Medical School.

connected to the oxygen cylinder. The oxygen pressure was regulated by means of a long handle on the main oxygen valve, and the pressure was read on a conventional high pressure gauge (10–140 atm.). Between one and 10 atmospheres the pressure was read on a special low pressure gauge, which could be shut off from the main line at pressures above 10 atmospheres. The "O" ring was always kept wet when under oxygen pressure and so far has not exploded.

The extinction of the blood at various oxygen tensions was read through a Wratten red filter, number 29F, and was compared with a constant gray filter held in a dry dummy-cuvette on the other side. This red filter gives the greatest separation between the oxy- and reduced hemoglobin. A gray filter (from a series of variously exposed photographic negatives) was chosen which gave a suitable reading for the oxygenated blood. As the light path on both sides was restricted to a diameter of one quarter of an inch, no wider diaphragm openings could be used

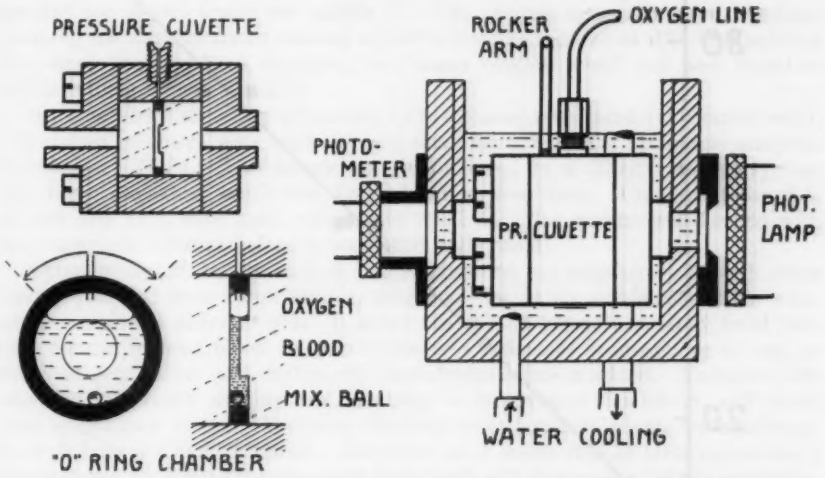


FIGURE 1. Cuvette arrangement for a study of high pressure dissociation curves.

than those corresponding to 0.50 on the extinction scale. The reading from full oxygenation to full reduction usually went from around 0.50 to about 1.20–1.30, giving a resolution of this extinction interval of about 2%.

Procedure

The heparinized blood, kept on ice, was strained, if necessary, through a few layers of gauze. Blood was transferred into three 20-cc. Erlenmeyer flasks, A, B, and C, 3.0 cc. in each. To A was added 0.3 cc. 0.17 *M* NaCl. To C the same amount of 0.17 *M* lactic acid solution was added very slowly while agitating the blood. To B was added 0.3 cc. of an equal mixture of 0.17 *M* NaCl and 0.17 *M* lactic acid. Samples A, B, and C hence contained 0, 8, and 16 millimols added lactic acid. This would drop the pH from A to B by approximately 0.7 pH units and from A to C by approximately 1.5 units. The flasks were provided with

stoppers pierced with a glass tubing 6 inches long, open at both ends. They were then rotated in a water bath of the desired temperature (10° or 4° C.). A fine suction tube was passed frequently into the flask through the glass tubing to renew the air. After about 20 minutes the blood would be well aerated. Sample A was taken out and its pH taken on a Cambridge Electron Ray pH Meter. The cuvette was provided with a water jacket to keep the blood at the desired temperature. If it was below about 7.4 a small known amount of NaOH would be added

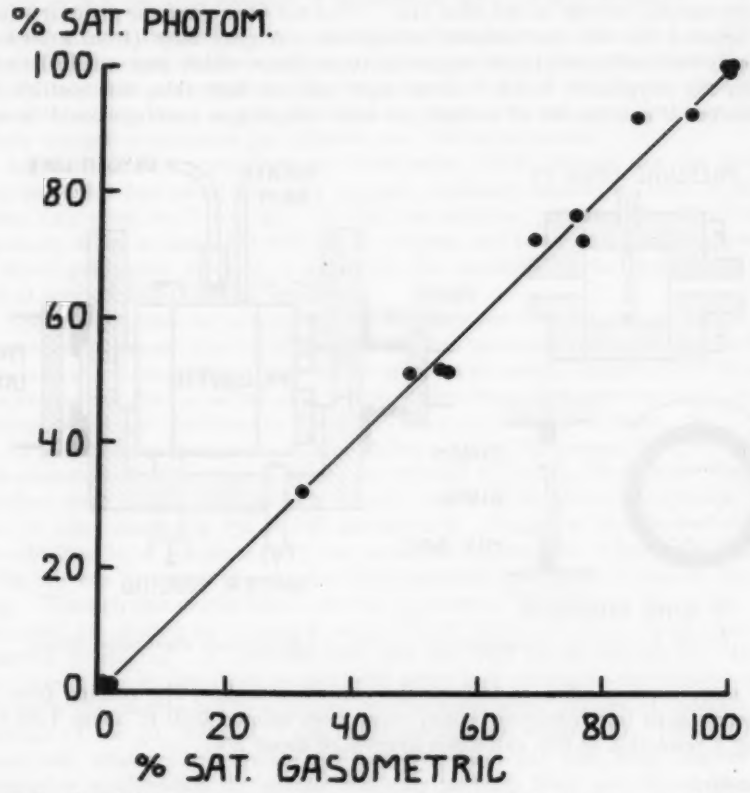


FIGURE 2. Correlation between optical and gasometric determinations of the oxygen saturation in the blood of tautog.

very carefully until a pH of about 7.6—7.8 was obtained. The flask was rotated again and the blood was then transferred to the pressure cuvette, which meanwhile had been temperature-equilibrated in its water bath on the photometer. Rocking of the cuvette was started, and the extinction was read as soon as a constant reading could be obtained. The oxygen line was flushed and connected with the cuvette and the pressure set to give one atmosphere O_2 in the cuvette (= 1.8 atm. total).

After about 5–10 minutes' equilibration the reading would be constant, and the pressure was raised to two atmospheres' O_2 (= 2.8 atm. total) and so on. Our oxygen contained less than 0.01% CO_2 and 0.02% N_2.

Meanwhile the remainder of sample A (about 2 cc.) was put in a rotating 50-cc. syringe, which was repeatedly flushed with helium (containing less than 0.01% O_2) for reduction of the hemoglobin. The helium was ejected and replaced four or five times, during a total equilibration time of about three quarters of an hour.

The pressure-equilibrated blood was removed from the cuvette by suction, and the cuvette thoroughly rinsed with cold 0.17 *M* saline. It was then flushed with helium and the reduced blood was introduced, completely filling the cuvette, without any contact with air. The rocker was started and the extinction of the reduced blood read.

Sample B was now taken from the equilibrator, part of it transferred to the pressure cuvette, and part of it to the pH meter. The whole procedure was then repeated for B and finally for sample C. The oxygen saturation was calculated by taking the fully saturated reading as 100% and the reduced as 0%, interpolating the intermediary readings according to a linear relation which had been found to hold for tautog blood (Fig. 2).

In cases where the effect of various CO_2 tensions was tested (at Bimini only), CO_2 and air mixtures were made up in a small spirometer and accurately analyzed (Scholander, 1947). The sample was equilibrated in a 50-cc. rotating syringe with this gas mixture, which was changed four or five times. Concentrations of 5, 10, and 20% CO_2 were used, accurate to $\pm$ 0.1%. The cuvette was flushed with the proper gas mixture before it was filled with blood.

Although optical determinations of the saturation are satisfactory enough when dealing with the same blood sample, they may not be so satisfactory when comparing samples of different pH. It is not too easy to get an accurately fixed zero point for the reduced blood. Hydrosulfite as a reductant is unsuitable to use, as the blood gets darker and darker the more hydrosulfite is added. Vacuum technique involves much shaking and handling of the delicate fish blood, and easily gives evaporation errors. We have obtained most constant results by equilibrating with helium (99.99% pure). But even so, it seems that at least sometimes a change in pH by careful titration with lactic acid will change the light transmission somewhat, due to such things as change (increase) in cell size or slight degree of hemolysis and fibrin formation. We do not believe that this source of error can have seriously distorted our results, but it is likely to be the cause of much of the spread in our data. In most cases the extinction readings of the reduced blood have matched each other within a few per cent at the different pH, and nearly always the fully oxygenated values have checked each other at different pH. The latter is the most important in the present connection.

As to the acidification of the blood we know in each case exactly the amount of acid added. The pH determinations were always made in aerated samples. A certain amount of drift and unsteadiness in the pH meter seemed unavoidable when it was used on our rolling moisture-laden ship, although the meter was kept in a dry-chamber. However, the fact that the drop in pH induced by the added known amounts of acid always came out with satisfactory regularity, as well as the fact that a colorimetric method used on our last cruise gave identical results,

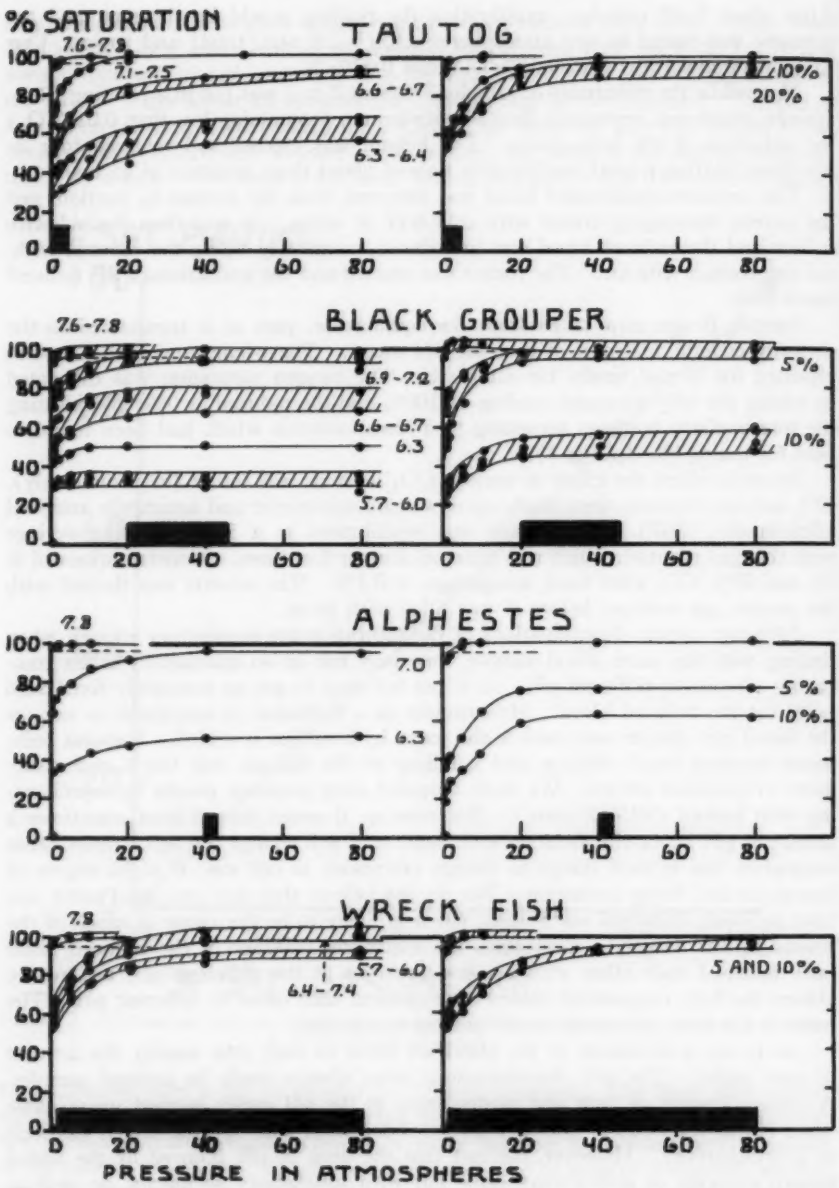


FIGURE 3. Oxygen saturation curves in various fishes at 10° C. Lactic acid was added to the blood in the curves on the left, CO_2 in those on the right. The pH values are given for curves of the lactic acid series. The equilibration pressure of CO_2 is given as per cent of one

lend assurance that our pH measurements must have been essentially correct. From very frequent checks back to our buffer standards, we consider our pH measurements to be reliable to within ± 0.1 pH unit.

Results

In Figures 3 and 4 the oxygen dissociation curves are plotted for fishes living from shallow surface waters to the abysmal depths. The curves span a range of pH from 8.0 to 5.6, and the carbon dioxide tensions used were 0.03, 5, 10, and 20% of an atmosphere.

It will be seen that in half of the eight species of deep sea fishes investigated (grouper, *Alphestes,* long-finned hake, ratfish) lactic acid in a concentration of about 100–150 mg. % (pH 6.7—5.6) produces a clear Root effect, in that the hemoglobin does not become more than 30–80% saturated at tensions as high as 140 atmospheres.[6] The curves run horizontal from above 20 to 30 atmospheres. In these cases, therefore, the Root effect could be responsible for the oxygen secretion.

In the remaining four deep sea species (rosefish, wreckfish, blue hake, long-nosed eel), however, even at pH 6 the hemoglobin became 90% or more saturated when the pressure exceeded 50–100 atmospheres. The arterial saturation at 0.2 atmosphere is no more than 90–95% at pH 7.6—7.8. Since the oxygen tension in the swimbladder frequently exceeds 50–100 atmospheres it appears impossible that the Root effect can account for gas secretion in these cases, unless possibly the pH falls below 5.6—6 in the blood vessels of the gland.

It seems dubious that the pH in the blood could ever reach such a low value as 6.0. If we consider pH 6.5 as the lowest physiological limit, then only the black grouper and possibly *Alphestes* would be able to maintain part of the hemoglobin in a reduced state in the presence of the high oxygen tension. Of course we do not know what the pH might be in the capillary blood passing through the gland. The lowest pH found in the blood of dead asphyxiated fishes was 6.9. At the highest acidity used *in vitro* it was impossible to avoid the precipitation of some fibrin, and hemolysis occurred with the addition of very little more acid.

If a high CO_2 tension were to be considered responsible for the oxygen secretion via the Root effect, then the CO_2 must be present at high concentrations in the blood vessels of the gland in which case it would diffuse with the oxygen into the bladder where its concentration can be measured. In shallow fishes swimbladder CO_2 is usually below 1–2% (Table I). In the wreckfish the oxygen secretion could not be explained by the Root effect unless the CO_2 were more than 10% of an atmosphere, and the tautog would require more than 20% CO_2 to secrete in deep water. Accurate determinations were made of the CO_2 tension in the swim-

[6] The Root effect was described from blood of shallow water fishes, dealing with oxygen pressures of no more than 700 mm. Hg (Root, 1931).

atmosphere. The broken line (90–95% saturation) denotes the arterial saturation at 0.2 atmosphere O_2 pressure. The solid bar on the base line is the range of depth in atmospheres where these species live. For each range of pH or CO_2, 3 or 4 separate runs with different bloods were usually made to get an impression of the individual variability. All curves were continued to 140 atmospheres' oxygen pressure, but showed no change from the values at 80 atmospheres.

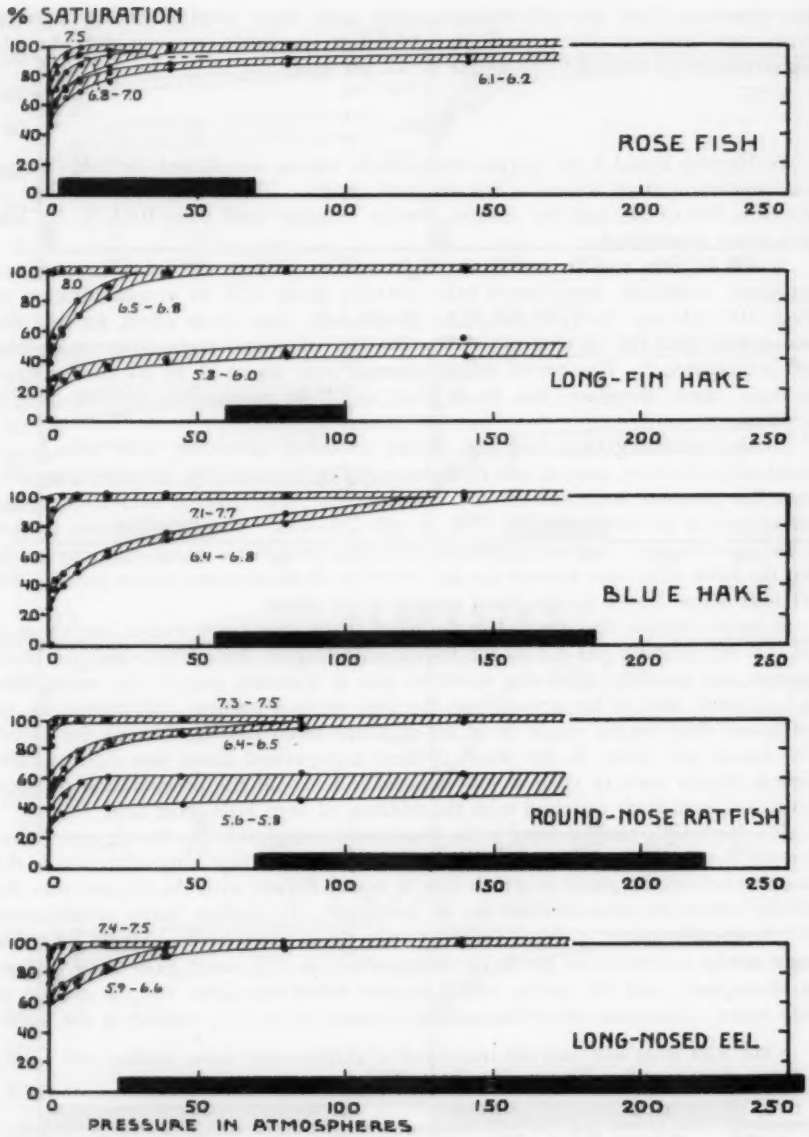


FIGURE 4. Oxygen saturation curves in blood of deep sea fishes at 4° C. The upper curve in each diagram represents the blood untreated, directly from the fish. In the other curves lactic acid has been added to give, after aeration, the pH stated for each curve. The solid bar on the base line is the depth range at which the fish lives. Each pH range was generally covered by two or three separate runs, using blood from different specimens. Arterial saturation would be no more than 90–95 vol. %.

bladder gas of the Bimini fishes (Table I, B), which were brought quickly to the surface. Assuming that only insignificant changes took place during the 4–7 minutes of getting the fish on deck, the tensions in the swimbladder gas were from 10–19% of one atmosphere CO_2 at the bottom. As the gas in the blown-up fish was dispersed all through its body cavity, it may well have picked up some CO_2 from the tissues.

If, as indicated by Root and Irving (1943), the CO_2 effect is simply a pH effect, then CO_2 cannot be the direct cause of the hypothetical oxygen dissociation from hemoglobin in our deep sea fishes. It was suggested by Black (1946) that HCl might be secreted rather than lactic acid. In tautog blood at pH 6.8 we found no greater Bohr effect with HCl than with lactic acid, and hence HCl does not seem to have any specific action beyond the pH effect.

In a discussion of the probable function of the *rete* it will be shown that in that connection, also, there are serious obstacles to regarding the Root effect as

TABLE I

Carbon dioxide content of swimbladder gas

Species	Depth of catch (m.)	Number of specimens	% CO_2 Low—High	Mean partial pressure of CO_2 $\left(\frac{\text{atm.}}{100}\right)$
A. Tautog (*Tautoga onitis*)	0–1	2	0.49–0.99	0.7
Queen triggerfish (*Balistes vetula*)	0–2	7	0.33–0.99	0.4
Blue parrotfish (*Scarus caeruleus*)	0–2	1	1.02	1.0
Black durgon (*Melichtys piceus*)	0–2	1	1.08	1.1
Sergeant major (*Abudefduf saxatilis*)	0–1	5	0.29–0.48	0.4
B. Black grouper	320–400	6	0.17–0.40	10
Alphestes	408	1	0.47	19
Wreckfish	640	1	0.22	14

Mean value for partial pressures of CO_2: Group A, 0.9% of 1 atm.; Group B, 14% of 1 atm.

the basic chemical reaction responsible for the oxygen secretion in deep sea fishes (Scholander, 1954).

We wish to express our gratitude to Dr. C. M. Breder, Jr., Chairman of the Department of Fishes, American Museum of Natural History, for arranging our stay at the Lerner Marine Laboratory. We are much indebted to Mr. Michael Lerner, who helped us with advice and generously supplied us with equipment and machinery for deep sea fishing. Mr. and Mrs. Marshall B. Bishop spared no effort in facilitating our work at the Lerner Marine Laboratory. We have benefited much from advice and stimulating discussions with Dr. A. C. Redfield of the Woods Hole Oceanographic Institution, and we are deeply grateful to Mr. W. C. Schroeder at the same institution for all possible cooperation on the deep sea cruises which he conducted off the New England coast. Mr. C. Grant and Mr. J. L. Allen gave invaluable help in the construction of the optical instrumentation, and Mr. K. Morrison and Mr. Stanley N. Eldridge in building the deck laboratory.

Mrs. Susan I. Scholander has given us much valuable assistance in the laboratory and the field and with the manuscript.

Summary

1. Oxygen dissociation curves at a P_{O_2} running from 0.2 to 140 atmospheres have been obtained from eight species of deep sea fishes, at a pH varying from 8.0 to 5.7 and at a CO_2 tension from 0.03 to 10–20% of one atmosphere.

2. The Root effect in acidified blood could be demonstrated in some of these species even up to 140 atmospheres, inasmuch as part of the hemoglobin remained unsaturated. In other species full arterial saturation occurred, however, at pH 6 or lower, at oxygen tensions much lower than those existing in the swimbladder. Hence the Root effect is not the mechanism for the oxygen secretion in deep sea fishes. If oxygen is derived from oxyhemoglobin it must be unloaded by some mechanism as yet unknown.

LITERATURE CITED

Black, V. S., 1946. Chloride, carbonic anhydrase, and catalase in the tissues of the perch, *Perca flavescens* (Mitchill). *Rev. Canadienne Biol.*, **5**: 311–318.

Copeland, D. E., 1952. The histophysiology of the teleostean physoclistous swimbladder. *J. Cell. Comp. Physiol.*, **40**: 317–335.

van Dam, L., and P. F. Scholander, 1953. Concentration of hemoglobin in the blood of deep sea fishes. *J. Cell. Comp. Physiol.*, **41**: 522–524.

Fänge, R., 1953. The mechanisms of gas transport in the euphysoclist swimbladder. Acta Physiol. Scandinavica, vol. 30, suppl. 110, 133 pp.

Goode, G. B., and T. H. Bean, 1895. Oceanic ichthyology. Smithsonian Contr. Knowledge, vols. 30–31.

Green, A. A., and R. W. Root, 1933. The equilibrium between hemoglobin and oxygen in the blood of certain fishes. *Biol. Bull.*, **64**: 383–404.

Haldane, J. S., 1922. Respiration. Yale Univ. Press, New Haven, Conn.

Hall, F. G., 1924. The functions of the swimbladder of fishes. *Biol. Bull.*, **47**: 79–126.

Hüfner, G., 1892. Zur physikalischen Chemie der Schwimmblasengase. Arch. Anat. Physiol., Physiol. Abth., 54–80.

Irving, L., and S. W. Grinnell, 1939. The secretion of oxygen at high pressure from the blood of fish. *J. Cell. Comp. Physiol.*, **14**: 409–410.

Jacobs, W., 1930. Untersuchungen zur Physiologie der Schwimmblase der Fische. I. Über die "Gassekretion" in der Schwimmblase von Physoklisten. *Zeitschr. Vergleichende Physiol.*, **11**: 565–629.

Jones, F. R. Harden, and N. B. Marshall, 1953. The structure and functions of the teleostean swimbladder. *Biol. Rev.*, **28**: 16–83.

Koch, H., 1934. L'émission de gaz dans le vésicule gazeuse des poissons. *Rev. Questions Sci., Ser. 4*, **26**: 385–409.

Krogh, A., and I. Leitch, 1919. The respiratory function of the blood in fishes. *J. Physiol.*, **52**: 288–300.

Ledebur, J. von, 1937. Über die Sekretion und Resorption von Gasen in der Fischschwimmblase. *Biol. Rev.*, **12**: 217–244.

Müller, J., 1840. Ueber Nebenkiemen und Wundernetze. *Arch. Anat., Physiol. Wissenschaftl. Med.*, 101–142.

Powers, E. B., F. G. Hopkins, T. A. Hickman and L. M. Shipe, 1932. The relation of respiration of fishes to environment. *Ecol. Monogr.*, **2**: 385–473.

Root, R. W., 1931. The respiratory function of the blood of marine fishes. *Biol. Bull.*, **61**: 427–456.

ROOT, R. W., AND L. IRVING, 1943. The effect of carbon dioxide and lactic acid on the oxygen-combining power of whole and hemolyzed blood of the marine fish *Tautoga onitis* (Linn.). *Biol. Bull.*, **84**: 207–212.

SAUNDERS, R. L., 1953. The swimbladder gas content of some freshwater fish with particular reference to the physostomes. *Canadian J. Zool.*, **31**: 547–560.

SCHOLANDER, P. F., 1947. Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. *J. Biol. Chem.*, **167**: 235–250.

SCHOLANDER, P. F., 1954. Secretion of gases against high pressures in the swimbladder of deep sea fishes. II. The *rete mirabile*. *Biol. Bull.*, **107**: 260–277.

SCHOLANDER, P. F., AND L. VAN DAM, 1953. Composition of the swimbladder gas in deep sea fishes. *Biol. Bull.*, **104**: 75–86.

STRITTMATTER, C. F., E. G. BALL AND O. COOPER, 1952. Glycolytic activity in the swimbladder gland. *Biol. Bull.*, **103**: 317.

TOWNSEND, C. H., 1901. Dredging and other records of the United States Fish Commission steamer Albatross. Rep. Commissioner, 1900, U. S. Commission Fish Fisheries, Part XXVI, 387–562.

WOODLAND, W. N. F., 1911. On the structure and function of the gas glands and *retia mirabilia* associated with the gas bladder of some teleostean fishes, with notes on the teleost pancreas. *Proc. Zool. Soc. London*, 183–248.

SECRETION OF GASES AGAINST HIGH PRESSURES IN THE SWIMBLADDER OF DEEP SEA FISHES.

II. THE RETE MIRABILE[1]

P. F. SCHOLANDER

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts, and Lerner Marine Laboratory, Bimini, Bahamas

The anatomical structure in the swimbladder of physoclist fishes associated with the gas secretion is the so-called red gland. This consists of a glandular, often button-like, structure exposed to the lumen of the swimbladder. The gland is provided with blood circulation through a so-called *rete mirabile* which consists of intermingled, parallel, unbranched, afferent and efferent capillaries. In deep sea fishes these may be more than one cm. long. In the outside layers of the swimbladder these capillaries unite to form the respective arteries and veins (Fig. 1). The structure as it varies in different fishes has been describd by Woodland (1911).

The essential feature from a functional standpoint seems to be that the *rete* represents a counter current exchange mechanism where the ingoing blood is brought into intimate diffusion contact with the outflowing blood (Müller, 1840; Woodland, 1911; Haldane, 1922; Hall, 1924; Jacobs, 1930; compare Scholander and van Dam, 1954). In principle the situation can be presented as in Figure 4.

It is obvious that to maintain a gradient of 100 atmospheres or more, the millimeter-thick swimbladder wall must be very impermeable to cut down diffusion losses. This seems to be accomplished by incorporating in the wall various kinds of solids (crystals and/or fibers). In the long-nosed eel the swimbladder is strikingly silvery.

At 100 atmospheres' O_2 pressure, one volume blood would dissolve four volumes oxygen. It is therefore clear that the blood flow leaving the swimbladder would cause an impossibly great loss of oxygen unless the tension in the blood were somehow reduced before it left the bladder wall.

The problem is similar to that facing a whale moving in ice water. How can it maintain body temperature, in spite of having the big flukes circulated? This is accomplished by having the fluke arteries completely surrounded by veins, so that the arterial heat is transferred to the veins and thus remains within the bulk of the body. This very general principle of heat exchange was described by Bazett *et al.* (1948) as applicable to the *venae commitantes* in man.

Let Figure 4, I represent a counter current heat exchanger where 0° water is flowing in. It is heated to 10° at the loop by some source and returns in intimate contact with the ingoing tubes. The heat exchange will then result in the establishment of a linear temperature gradient in the tubes, as pictured (Fig. 4, II). The temperature of the leaving water would depend upon the efficiency of the heat

[1] Contribution No. 700 from the Woods Hole Oceanographic Institution. The investigation was supported by a grant from the National Science Foundation.

exchanger, and might be one degree. If the effluent water were run out separately, isolated from the influent water, the temperature would have been 10°. Hence a ten-fold saving of heat was accomplished by the counter current exchange mechanism. If the temperature of the source of heat is fixed, it should be noted that the loop could only approach this temperature, but could not reach or exceed it.

The same situation would also obtain in regard to a diffusion exchange system in which instead of temperature we deal with gas tensions in a liquid.

FIGURE 1. Schematic drawing of capillary *rete* and gland in relation to the swimbladder wall, which is represented with exaggerated thickness. To the left the *rete* ends in the gland (*e.g.*, in *Antimora, Coryphaenoides*); to the right blood vessels radiate from the gland over the surface of the swimbladder (*Sebastes, Synaphobranchus*).

MATERIAL

The deep sea material used in the present investigation was obtained from the Lerner Marine Laboratory, Bimini, and from cruises with the dragger *Cap'n Bill II* off the New England coast. (For particulars see Scholander and van Dam, 1954.)

HISTOLOGICAL STRUCTURE OF THE RETE

Sections of the *rete* from the long-nosed eel and the rosefish are given in Figure 2 (A-F). The *rete* of the rosefish is similar to that found in the common eel described by Krogh (1919). By differential injection he showed that the afferent smaller capillaries surround the larger efferent venous capillaries much in the same manner as is seen in the rosefish (D). In the ratfishes (C) these small capillaries surrounding the bigger ones are thick-walled and clearly arterial. In the long-nosed eel (A) both afferent and efferent capillaries tend to be squarish, about 13 $\times$ 13 μ. The walls between the capillaries average 1.5 μ. Most of the capillaries containing red cells are surrounded on all four sides by empty capillaries. It is believed from this that one *rete* was empty and that the *retes* are fitted together like the squares on a checkerboard.

The checkerboard arrangement is the geometrical arrangement which gives the maximal diffusion exchange between the afferent and efferent capillaries, and it is remarkable that we find it in our deepest fish. The only other solution to the topological problem of making four polygons (black or white) meet at one point in such a way that black always borders white is realized in the hexagonal star pattern found in the *rete* of the rosefish (D).

It would seem that the checkerboard pattern might have developed through a non-staggered columnar arrangement of the primary vessels (Fig. 3a), whereas the star pattern might have developed from a staggered pattern of the primary

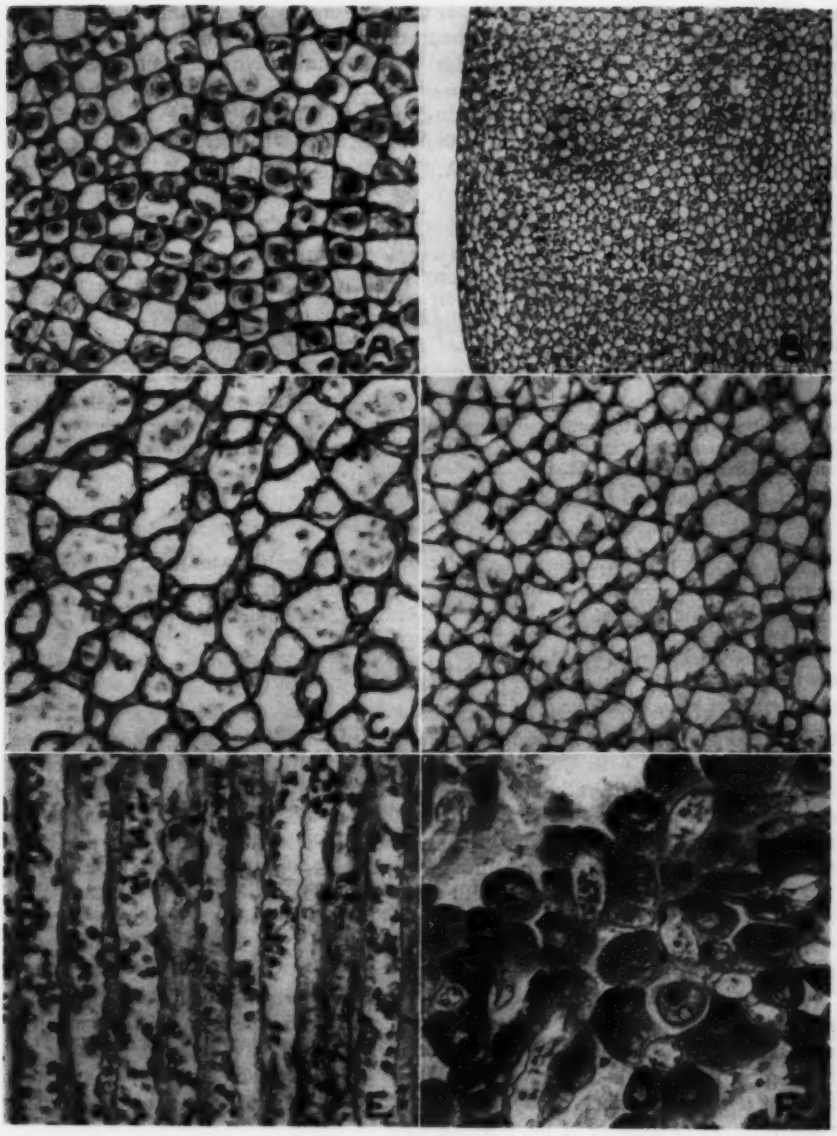


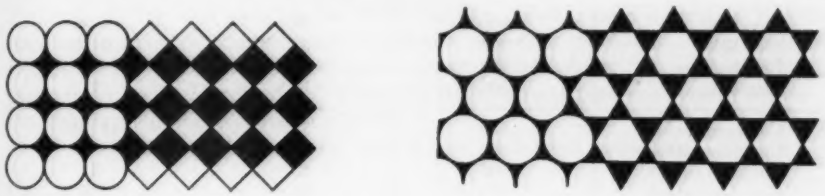
FIGURE 2. Sections through *rete* in deep sea fishes. A: *Synaphobranchus*, cross section × 300. Each polygon (square) is a single capillary. B: Same as A, × 100. C: *Coryphaenoides*, cross section × 300. D: *Sebastes*, cross section × 300. E: *Coryphaenoides*, longitudinal section × 300. Many red cells in capillaries. F: *Coryphaenoides*. Glandular epithelium from

vessels (Fig. 3b). In both cases the arterial capillaries may have developed in the interstices between the veins.

In many *retes* the loops of the glandular buttons arise from a vascular plexus located at the base of the buttons. This must naturally be so in all cases where the arterial *rete* capillaries outnumber the venous capillaries, as in the common eel, or in the rose- and ratfishes.

The length of the *rete* capillaries varies somewhat with the species, and increases generally with the depth. Measurements are given in Table I.

Figure 2F shows the surface layers of the gland "button," with capillaries surrounded by the thick glandular epithelium.



a b

FIGURE 3. a. Possible development of the checkerboard pattern of the *rete* in *Synaphobranchus*, the primary capillaries (veins) in non-staggered rows with the arterial capillaries in the interstices. b. Possible development of *rete* in rosefish, the primary capillaries (venous) in staggered rows with the arterial capillaries in the interstices, resulting in the hexagonal star pattern.

DIFFUSION CHARACTERISTICS

From the structure and dimensions of a *rete* such as in the long-nosed eel, we may estimate what diffusion characteristics a given *rete* may have.

Let us take as unit a rete 1 cm.^{2} in cross section and 1 cm. long with capillary characteristics of the deep sea eel. The capillaries average 13 μ each side, making the circumference 52 μ. Length is 1 cm. and hence the surface area of each capillary tube is 0.0052 cm.^{2} Due to the checkered arrangement all of this surface is available for diffusion. The wall between two capillaries averages 1.5 μ. There are about 500,000 capillaries per cm.^{2} Using a diffusion constant of 150 mm.^{3}/μ/cm.^{2}/min./atm. (Krogh, 1919, p. 195), we find that the diffusion across the length of this one capillary is then 0.52 mm.^{3}/min./atm. per capillary. There are 250,000 pairs of capillaries in this *rete*, and hence the total diffusion, Q, is 130 cc./min./atm.

SYMBOLS AND BASIC EQUATIONS

It is of interest to formulate a few relations pertaining to such a counter current system in order to be able to estimate more closely what a fish *rete* might be able to do.

"button," × 225. Several capillaries are seen buried among the large glandular cells. (Staining: A, B: Gomori's chrome alum-hematoxylin and phloxine, Bouin. C: Periodic acid-Schiff, Bouin. D: Periodic acid-Schiff, 80% alcohol. E: Hematoxylin and phloxine, Bouin. F: Gomori's chrome alum-hematoxylin and eosine, Bouin.) Sections and photographs by courtesy of Dept. Anatomy, Harvard Medical School.

TABLE I

Size of fish versus length of rete mirabile

Species	Depth of catch (m.)	Length	
		Fish (cm.)	*Rete* (mm.)
Rosefish	600	46	7–9
Rosefish	600	45	8–9
Rosefish	600	32	7–8
Rosefish	600	32	7–10
Rosefish	600	32	8–10
Blue hake	900	38	7–8
Blue hake	900	37	9–10
Blue hake	900	17	9
Blue hake	900	10.5	9
Common ratfish	600	34	11–13
Common ratfish	600	28	8–9
Common ratfish	600	18	8
Common ratfish	600	16	7–8

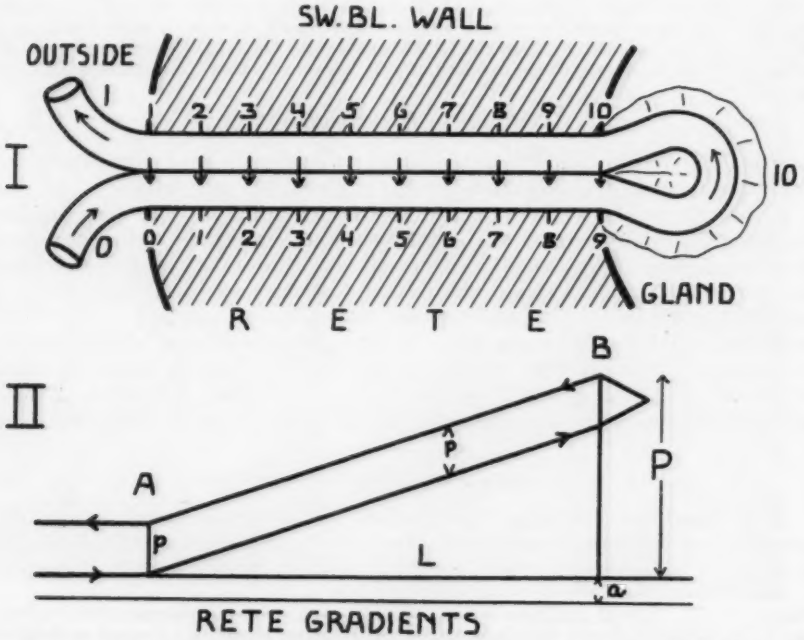


FIGURE 4. Schematic presentation of the counter current exchange principle in the swimbladder. I. Afferent and efferent capillaries. The numbers illustrate the linear gradient set up from the inside to the outside of the structure. The arrows signify the constant trans-*rete* diffusion gradient. II. Graphic representation of the gradients. L is length of the *rete;* p is trans-*rete* gradient; P is gas tension in the swimbladder; a is gas tension of inflowing blood.

Hargitay and Kuhn (1951) have developed an extensive physical treatment of this principle in relation to its probable applicability to the kidney function. Their "hairpin counter current multiplier system" is the loop of Henle. The primary concentration difference which is augmented by the system is discussed on the assumption of a semi-permeable membrane separating the afferent from the efferent flow. The difference in hydrostatic pressure due to flow presses water through the membrane and results in the primary concentration difference.

In the fish *rete* we are dealing with a different situation, inasmuch as it may be assumed that the capillaries are extremely permeable to lipid-soluble substances, especially oxygen and carbon dioxide (Pappenheimer, Renkin and Borrero, 1951), very much more so than to water. Hence it is possible to discuss the diffusion exchange in the *rete mirabile* on much simpler terms, namely, relative to what happens to gases or solutes which are simply added to the blood. The treatment is presented, realizing that it is grossly oversimplified, and at the same time it is a pleasure to credit Hargitay and Kuhn for their introduction and interpretation of the hairpin multiplier system in biology.

The following symbols will be used in the subsequent discussion:

Length of rete	= L cm.
O_2 pressure in blood entering afferent *rete*	= a atm.
Trans-*rete* gradient (O_2 tension gradient between afferent and efferent *rete*)	= p atm.
O_2 equilibrium pressure in swimbladder	= P + a atm.
O_2 pressure in blood leaving efferent *rete* (A)	= p + a atm.
Blood flow	= V cc./min.
Trans-*rete* diffusion	= Q cc./min./atm./cm.
Solubility coefficient for oxygen in blood	= α cc./cc./atm.
O_2 secreted from blood	= D cc./cc.
O_2 loss by respiration and diffusion	= R cc./min.

Based on the present fragmentary knowledge it seems possible that the oxygen can be secreted in two different ways: (a) molecular oxygen is directly split off from some compound in the blood, (b) oxygen in some form is transported to the glandular cells and stored there as a compound ready for intermittent use. These two possibilities will be dealt with separately.

a. *Release of oxygen directly from the blood.* It is here assumed that some constituent, at the present unidentified, is capable of dissociating off free oxygen in the blood against very high oxygen tensions. As blood cannot be stored in the gland to any significant degree, the release of oxygen stands in direct time relation to the blood flow. The fraction of the oxygen which is dissociated off and which stays dissociated off is called D and is without any connotation as to what is the nature of the responsible compound.

The diffusion characteristics of the *rete* may now be formulated in two basic equations (Fig. 4 II).

$$P\alpha V = pQ + p\alpha V \tag{1}$$

$$DV = p\alpha V + R \tag{2}$$

Equation 1 states that the amount of physically dissolved oxygen entering the effluent *rete* per minute (at B, Fig. 4) equals a fraction diffusing across the *rete* plus a fraction leaving the effluent *rete* (A). Equation 2 states that the amount of bound oxygen to be secreted, which enters at A, equals the amount of physically dissolved oxygen leaving at A plus an amount of oxygen lost from the swimbladder by other avenues (respiration, diffusion loss, etc.).

(i) *Quantitative evaluation of the rete function.* The oxygen secretion will first be dealt with without considering the term R. In equation 1 the term $p\alpha V$ is negligibly small compared to $P\alpha V$ and can be omitted. We then get the following relation:

$$\left.\begin{aligned} P\alpha V &= pQ \\ DV &= p\alpha V \end{aligned}\right\} \tag{3}$$

and therefore

$$P = \frac{DQ}{\alpha^2 V} \tag{4}$$

The equilibrium pressure P is consequently inversely proportional to the blood flow and directly proportional to the amount (D) of dissociated oxygen per cc. blood. P is also proportional to Q and hence to the length of the *rete*. This is borne out by the observation that for a given depth and for a given species both small and large individuals had the same length of *rete*, although the cross sections varied markedly with the size (Table I). One might make an analogy between length of *rete* and voltage, between cross section of *rete* and current (amps.).

We shall consider the *rete* equations in relation to a hypothetical but plausible deep sea fish with the following constants:

Weight	1000 g.
Swimbladder volume	50 cc.
Oxygen consumption, 8° C.	1 cc./min.
Respiratory need of swimbladder and other losses = R	0.01 cc./min.
Oxygen secreted from blood = D	0.05 cc./cc.
Oxygen solubility coefficient = α, 8° C.	0.04 cc./cc.
Trans-*rete* diffusion = Q	130 cc./min./atm.

Q varies greatly from species to species and can be given great latitude.

Let us assume a blood flow (V) through the *rete* capillaries of 1 cc. per minute and that 0.05 cc. O_2 per cc. blood (= D) is dissociated off by action of the gland. The oxygen secretion would then amount to only 50 mm.^{3}/min. or 5% of the total oxygen consumption. According to equation 4 the equilibrium pressure would then be no less than 4060 atmospheres. Obviously, therefore, if there are no losses and if the dissociation is not pressure-sensitive, the potential pressures are enormous, and would increase with lowering of the blood flow.

It is necessary to introduce a term (R) expressing the loss of oxygen by such factors as the respiratory need of the gland and the lining of the swimbladder, diffusion loss, and oxygen used for compression work.

The equations would then be:

$$\left.\begin{aligned} P &= p\frac{Q}{\alpha V} \\ DV &= p\alpha V + R \end{aligned}\right\}$$

or

$$P = \frac{(DV - R)Q}{\alpha^2 V^2} \tag{5}$$

It is expedient at this point to consider what rates of oxygen deposition might be pertinent.

Bohr (1905) found that cod could fill its swimbladder with oxygen in about one day. Akita (1936) found the same rate for *Monacanthus*. Copeland (1952) found that *Fundulus* would need about two days, if it were able to maintain full rate for so long. If the cod and *Monacanthus* were to fill their bladders at 100 atmospheres' pressure it would take 100 days to do it. Such a drastic thing is hardly relevant for a deep sea fish. It may, however, have to compensate for a buoyancy change of, let us say, 10%, by moving from 900 meters' depth down to 1000 meters. This would require 10 days of secretion. The quantitative development of the *rete* varies greatly from species to species. Suppose that our fish can do twice as well as the cod. It would then need five days to accomplish the 10% buoyancy change and would secrete at a rate of 100 cc. oxygen of one atmosphere a day, or approximately 70 mm.³/min. This would represent 7% of the total metabolic rate of the fish. Let us assume that the respiratory and diffusion loss (R) adds to it 10 mm.³/min.[2] This would then give a total release of 80 mm.³/min., which would require that 1.6 cc. blood per minute enter the *rete*. The potential pressure developed using these figures in equation 5 would come out as 2220 atmospheres.

When the necessary amount of gas has been deposited to accomplish the buoyancy change the fish must shut down the deposition to a simple maintenance level. This may very possibly go via a reduction of R, which may drop to, let us say, 5 mm.³/min. This, according to equation 5, would make the blood flow (V) very slightly more than 0.1 cc./min., *i.e.*, 16 times less than at full secretion rate. During activity the blood vessels of the gland dilate markedly (Hall, 1924; Fänge, 1953). A less economical way of maintaining constant swimbladder volume at 100 atmospheres (or of reducing the volume) would be to draw some blood off through the non-*rete* system, like the capillaries of the "oval." Then 67 mm.³/min. of O_2 would have to be run off from the swimbladder, which would require that 4 mm.³ blood per minute be bled through the oval.

(ii) *Amount of oxygen secreted per cc. blood at steady state.* By considering the maximal rate at which oxygen can be deposited in the swimbladder one may calculate D, *i.e.*, the amount of dissociable oxygen carried in by the blood.

If, again, 80 mm.³/min. is taken as the maximum rate of secretion and if the

[2] The work of compressing one mol gas from 0.2 to 100 atmospheres at 8° is equal to $1.99 \times T \times 2.3 \log (P_1/P_2)$ Cals. = 3.5 Cals. The caloric equivalent of one liter O_2 equals 4.8 Cal. or 104 Cal. per mol. Hence for each part of oxygen secreted into the swimbladder a minimum of 3% goes to the work of compression, or 2.1 mm.³ out of the above 10 mm.³ which constitute R.

blood flow (V) through the *rete* is 2 cc./mm., the blood would have to unload 40 mm.^{3} oxygen per cc. blood, or more or less what the hemoglobin could do if dissociated.

The amount of physically dissolved oxygen in the blood is no more than $0.04 \times 0.2 = 0.008$ vol. % and therefore cannot be the source of a steady-state secretion, even if the solubility coefficient in the effluent blood were drastically lowered by some unknown process. The tension difference (p) between affluent and effluent blood could only be a few millimeters Hg.

b. *Secretion of oxygen from the gland cells.* Considering that we have failed to identify hemoglobin or any other compound in the blood as the direct source for the secreted oxygen, we must consider the possibility of a cellular secretion, where the oxygen is ultimately transported to the glandular cells via some compound in the blood. This compound, or a derivative thereof, might be stored in the epithelial cells for intermittent use. Non-steady-state events are conspicuously associated with the secretion. Glycogen is thus stored in large quantities by the gland cells if secretion is inhibited. When the gland is secreting, the glycogen is being used, and secretion cannot go on when the store of glycogen is depleted (Copeland, 1952; Fänge, 1953). If a breakdown of a stored compound is what determines the maximal rate of oxygen secretion, then the amount (D) of oxygen transported to the gland per cc. blood can be much smaller than would be necessary if the oxygen is released directly from the blood. From the information available it is impossible to decide between cellular secretion of oxygen and the release of molecular oxygen directly from the blood.

Conclusions. The discussion of the *rete* may be summarized in the following statements. The structure of the *rete* is such as to provide for an extremely efficient counter current diffusion exchange mechanism between inflowing and outflowing blood. This makes possible a steep tension gradient within the *rete* so that tension loss through the circulation becomes very low. Quantitative considerations of the diffusion efficiency of the *rete* make it clear that the limiting equilibrium pressure theoretically could reach tremendous values, provided the dissociation pressure of the underlying chemical or physical reaction is even higher. If oxygen cannot be chemically stored in the gland it must enter through the blood concomitantly with the secretion. This would require an amount of oxygen per cc. blood larger than that physically dissolved in the arterial blood. The nature of the responsible compound is not known.

The Role of Lactic Acid and CO_2

We have seen that thanks to the multiplication principle of the *rete,* small primary concentration effects can build up to considerable pressures, limited ultimately by the pressure characteristics of the primary chemical or physical dissociation. It was shown that the Root effect or Bohr effect at pH 6 was nullified already at 50 atmospheres in some fishes which live and secrete oxygen at much greater depths, and therefore it could not be the primary pressure reaction. Whether stronger lactic acid and lower pH could do it we do not know. It seems difficult *in vitro* to go much further without irreversible damage to the blood (Scholander and van Dam, 1954).

As regards CO_2 it was likewise shown that in some fishes 5 and 10% CO_2 would

be inadequate to dissociate off oxygen at the required pressures. The question that arises is: Could the CO_2 tension be higher than this, and if so what would be the pressure characteristics of the Bohr or Root effect?

The CO_2 content of the aerated blood of several deep sea species is given in Table II. If a small amount of lactic acid were added to the blood by the gland the CO_2 pressure would build up in the *rete* and the swimbladder, and the approximate tension gradients can be calculated, inasmuch as the diffusion constant can be taken as about 25 times that of oxygen (Krogh, 1919), and the solubility coefficient is about 1.0, or again 25 times that of oxygen. This would make Q of equation 1 for $CO_2 = 25 \times 130 = 3250$ cc./min./atm.

If the CO_2 effect were responsible for the oxygen secretion in the wreckfish we would expect to find in the swimbladder at all times of steady-state a CO_2 tension higher than 10% of an atmosphere. A wreckfish caught at 640 meters had 0.22% CO_2 or 14% of an atmosphere. Six black groupers had from 6.6—13% of an atmosphere CO_2. In contrast to this 27 specimens of surface fishes, comprising 6

TABLE II

*Carbon dioxide content of blood**

Species	Depth of catch (m.)	Number of specimens	CO_2 (vol. %)	
			Range	Mean
A. Undiluted whole blood, aerated at 10° C.				
Black grouper	333–375	4	4.8–6.3	5.5
Alphestes	410	1	—	7.0
Wreckfish	640	2	6.8–8.0	7.4
B. Whole blood plus lactic acid 0.17 *M* (10:1), aerated at 10° C.				
Black grouper	333–375	2	1.4–1.5	1.45
Alphestes	410	1		1.9

* Method of Scholander, Flemister, and Irving (1947), modified.

species, had a CO_2 tension averaging only 0.7%. It has been shown by Jacobs (1934) and von Ledebur (1937) in 10 species of physoclist fishes, which were induced to secretion by puncture, that on the average the CO_2 tension in the swimbladder rose by only 1.4—3.1% while the O_2 rose to 80–90%. In some of the species the CO_2 did not rise at all. A *Serranus cabrilla* with the oval obliterated accumulated no more than 3% CO_2 during the process of secretion. The relatively high CO_2 tensions in deep sea fishes may hence very likely be artifacts, as CO_2 diffuses so fast. If, nevertheless, we assume that our deep sea fish were to require as much as 0.5 atm. CO_2 in the swimbladder [3] to dissociate off the oxygen, and if we assume this Root effect to be reversible when the CO_2 pressure decreases, then we can estimate the rate at which such deposition could take place.

If $P = 0.5$ atm., $Q = 3250$ cc./min., αCO_2 is 1.0, and at a high rate of secretion $V = 1$ cc./min., then according to equation 3

$$p = 0.12 \text{ mm. Hg}$$

[3] Tautog blood begins to hemolyze at one atmosphere CO_2.

In other words the P_{CO_2} of the "venous" blood leaving the *rete* would be only 0.12 mm. Hg higher than that of the "arterial" blood entering the *rete*. Now the oxygen *tension* of the leaving blood is always slightly higher than the tension of the entering blood. The leaving hemoglobin would hence be nearly, if not completely, arterialized. If under these circumstances any oxygen at all were deposited it would be at an infinitesimally small rate.

If the leaving CO_2 tension were to be high enough to effect a half-saturation of the hemoglobin (which is necessary for the secretion rate), one would expect at least around 10 mm. Hg CO_2 tension in the leaving blood. This would, however, require some 43 atmospheres' CO_2 tension in the swimbladder, which of course is absurd.

We see therefore that unless CO_2 at tensions higher than we tested produces some *irreversible* oxygen-splitting from the hemoglobin, we cannot accept the CO_2 effect as producing the oxygen secretion.

Koch (1934) considered that if the elevated CO_2 tension passed onto the arterial side of the *rete*, the O_2 would increase there and O_2 would diffuse across to the venous side, which would seem like a short circuit with regard to the O_2. In order to avoid this he thought that buffer might diffuse across from the arterial side to the venous, due to hydrostatic forces. If this were to happen it would at best mean a lower P (CO_2) on the venous side, hence an even earlier recombination of oxygen with the hemoglobin before the blood left the *rete*, and hence no secretion.

According to either of the two theories (CO_2 and lactic acid) the oxygen is liberated directly from the blood and hence *in* the *rete*. This obviously wastes part of the *rete* as a diffusion barrier, and the oxygen must also diffuse from the *rete* into the swimbladder. Here we encounter another seemingly backwards situation, inasmuch as in many fishes the capillary loops in the gland are covered with a deep layer of epithelial cells (Fig. 2F), which must hinder very substantially the gas diffusion into the swimbladder. The loops are also usually very much fewer than the *rete* capillaries. In order to get an efficient gas exchange we would expect rather a great many almost naked capillaries, such as in the lungs.

The anatomical arrangement seems to fit a cellular gas secretion much better. This would leave the whole *rete* as a simple counter current diffusion barrier against oxygen loss, and the secreting cells covering the capillary loops would aid the *rete* as a diffusion barrier, rather than counteract it.

There are grave difficulties in seeing the Bohr or Root effects as the cause of the secretion, and at the present time it seems indicated that many observed events associated with the secretion of oxygen, such as the occasional large rise in CO_2 (Jacobs, 1930; von Ledebur, 1937; Rostorfer, 1942), the acid formation (Hall, 1924), the glycogen disappearance (Copeland, 1952; Fänge, 1953), carbonic anhydrase activity (Leiner, 1938, 1940; Copeland, 1952; Fänge, 1953), potential glycolytic activity, lactic acid formation (Strittmatter, Ball and Cooper, 1952; Fänge, 1953) must be fitted into the secretion picture in some other way.

If dissociation of oxyhemoglobin still is to be considered as the direct cause of the pressure build-up, it must be brought about by a diffusible x-substance (from the gland cells), which would dissociate off oxygen against much higher pressure than does lactic acid. This x-substance would either have to leave with the blood and must then be quite indiffusible through the *rete* capillaries, in contrast to the

gland capillaries, or if easily diffusible it would have to effect an irreversible reaction, such as methemoglobin formation, to be repaired outside the gland. Such a substance and such diffusion conditions are purely hypothetical, although perhaps possible.

There are at the present time unresolved obstacles in regarding the Root or Bohr effect as the secretion mechanism. It has not been proved that hemoglobin is involved, and it would be premature to disregard the possibility of a cellular oxygen secretion.

Conclusions. In several deep sea fishes the blood acidified with lactic acid to pH 6 or lower arterializes fully at a much lower oxygen pressure than that existing in the swimbladder. Hence lactic acid via the Root effect cannot be the cause of oxygen secretion. If the CO_2 effect were to be the cause of the secretion, CO_2 would have to leave the gland at a pressure of at least 5–10 mm. Hg. This would

TABLE III

Nitrogen content of the swimbladder gas in deep sea fishes

Species	Depth of catch (m.)	Number of specimens	% N_2	Mean partial pressure of N_2 (atm.)
Black grouper	320–400	6	12.5–16.8	5.4
Alphestes	410	1	18.5	7.6
Wreckfish	640	1	11.0	7.0
Rosefish	660	2	20.0	13.2
Long-finned hake	1300	5	3.0–3.7	4.4
Blue Hake	1240	4	6.0–7.6	8.0
Round-nosed ratfish	1100	3	9.4–10.0	10.8
	1200–1240	3	8.4–10.3	11.6
Common ratfish (*Macrourus bairdii*)	1240	4	7.2–7.8	9.3
Long-nosed eel	1200	1	7.6	9.1

require a CO_2 pressure in the swimbladder of 20–40 atmospheres, which is out of the question. If hemoglobin is directly involved in the oxygen secretion the mechanism by which it is dissociated is unknown. One is led to consider seriously the possibility of a cellular oxygen secretion.

SECRETION OF INERT GASES

It has been shown that the nitrogen tension found in the swimbladder of marine fishes is practically always higher than in the sea water. In each species the nitrogen is deposited as a relatively constant proportion of the oxygen, but the fraction varies from species to species. It usually lies between 2 and 15%. In deep sea fishes the N_2 tension can therefore reach as much as 15–20 atmospheres. New data from greater depths confirm these conclusions, Table III and Figure 5. It does not seem possible to explain the high nitrogen as an artifact (Scholander and van Dam, 1953).

The whitefish (Hüfner, 1892) and several more species of freshwater physo-

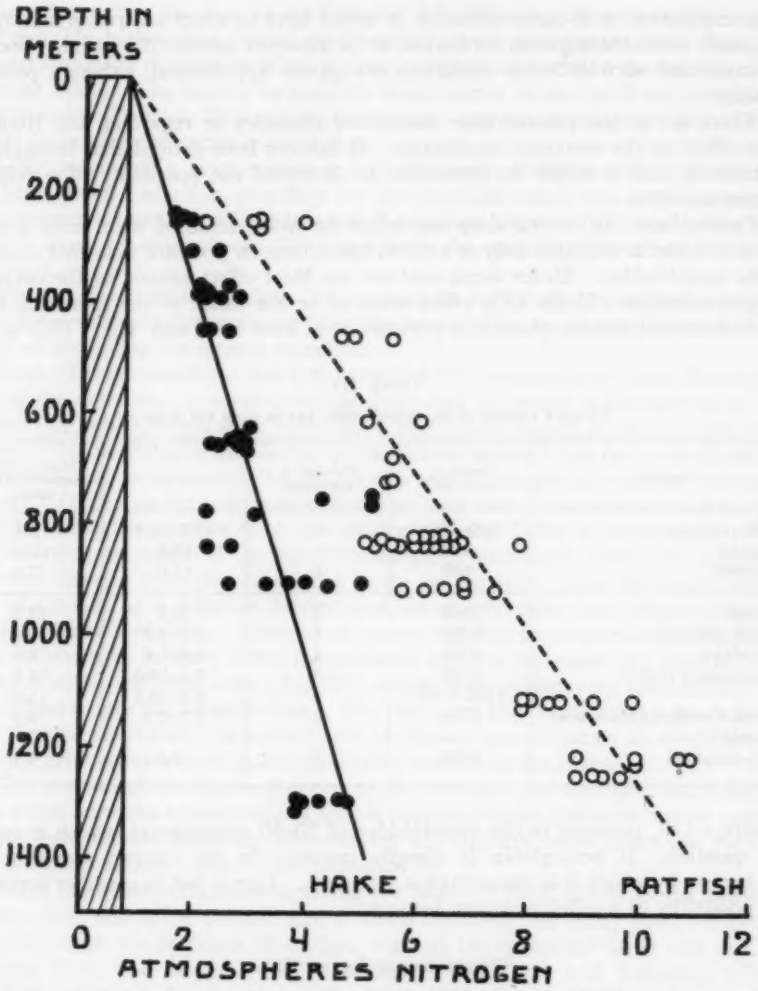


FIGURE 5. Relation of nitrogen tension in swimbladder to depth for the long-finned hake (*Urophycis chesteri*) and common ratfish (*Macrourus bairdii*).

stomes (Saunders, 1953) are able to deposit "pure" nitrogen against some 6–8 atm. pressure.

The argon-nitrogen ratio in the swimbladder. An important clue as to how basically nitrogen enters the swimbladder can be obtained by comparing the argon-nitrogen ratio in the swimbladder gas to the ratio in the air, as pointed out by Koch (1934). He considered, and rightly so, that the fact that the A/N_2 ratio found by

Schloesing and Richard (1896) was near the atmospheric ratio indicated that the nitrogen could not have been chemically secreted.

Some of our deep sea samples have been analyzed for argon. This was made possible through the courtesy of Dr. A. O. C. Nier and of Mr. Bailey Donnally, who kindly made the analyses for us on the mass spectrometer. The results will be seen in Table IV and are in favor of a physical deposition of nitrogen. If nitrogen were secreted chemically, the argon/N_2 ratio would be decreased in the given fishes between 6 and 20 times, *i.e.*, it would have been found to be between 0.2 and 0.06, instead of 1.18. Although there are substantial deviations from the air ratio they are certainly not of the order which would suggest nitrogen secretion by chemical means. Considering the fact that the argon may be expected to have

TABLE IV

Argon-nitrogen ratio in the swimbladder gas of deep sea fishes

Species	Depth of catch (atm.)	% (N_2 + A)	N_2 + A atm.	$\frac{100A}{N_2}$
Air	1	80	0.8	1.18
Blue hake		6.2		1.16
Blue hake		6.0		1.22
Common ratfish	80	7.2	5.8	1.00
Common ratfish	80	7.6	6.1	1.61
Common ratfish	80	7.4	5.9	1.08
Rosefish	50	20.0	10.0	0.94
Rosefish	50	20.0	10.0	0.87
Long-nosed eel*	167			1.94
Moray eel*	9			1.85
Air*	1			1.18

* Schloesing and Richard, 1896. Long-nosed eel, *Synaphobranchus pinnatus*; Moray eel, *Muraena helena*.

twice the diffusion coefficient of nitrogen, one would expect the argon fraction to tend to be low rather than high (Scholander and van Dam, 1953).

There are unfortunately no data available on the argon/N_2 ratio in the whitefish, nor on the presence of organic gases, which would appear as nitrogen.

THEORIES FOR SECRETION OF INERT GASES

Two theories have been offered to explain the high nitrogen tensions.

a. *The bubble theory.* Powers *et al.* (1932) assumed that the Root effect would result in the formation of bubbles and that these when entering the swimbladder would transport nitrogen in from the surroundings. If the Root effect were responsible, the highest possible local pressure increase would be in the blood, and the bubbles would form there, and as Koch (1934) pointed out, it is hard to see how they would get out of the blood. Haldane (1922) pointed out that "bends" in human divers occur only if the pressure is diminished by more than 50% at a time. The highest increase in the plasma which dissociation of 9 vol. % oxygen

could give is 3 atmospheres. Operating with blood equilibrated at 100 atmospheres' oxygen pressure in our cuvette, bubbles never formed at sudden drops of pressure, even ten times 3 atmospheres. Hence in deep sea fishes gas bubbles cannot very well form in the capillaries of the gland or of the *rete*. Experimentally, gas bubbles have not been observed to form within living cells, even after decompression from very high gas pressures (Harvey *et al.*, 1944). In shallow fishes, however, gas bubbles were seen to form in the mucus covering the gland when the gland was stimulated to activity (Fänge, 1953).

b. *Secretion by change in the solubility coefficient.* The second theory, proposed by Koch (1934), seems to offer a possible mechanism for the increase in nitrogen and inert gases. He considered the possibility that by some means the blood in the swimbladder vessels would have a lower solubility coefficient for gases than it does outside the swimbladder. This could come about by an increase in the temperature of the active gland, or by added solutes.

Any metabolic heat gradient would certainly be very small, as the swimbladder wall has no aspect of being a thermal insulator. Due to the extraordinry exchange capacity of the *rete* there would be, if any, only an infinitesimal difference in the temperature of blood entering and leaving the *rete*. Hence they would have the same nitrogen capacity and N_2 deposition could then not occur (equation 6).

If the solubility were depressed by the addition of some solute entering the blood from the gland, the nitrogen tension would slightly increase on the venous side. If this situation persisted so that the blood would leave the *rete* with a decreased solubility coefficient the following relation would obtain, using the symbols given above and essentially equations 1 and 2:

$$\left.\begin{aligned} P\alpha_2 V &= pQ \\ a\alpha_1 V &= (a + p)\alpha_2 V \end{aligned}\right\} \tag{6}$$

$$P = \frac{a(\alpha_1 - \alpha_2)Q}{\alpha_2^2 V} \tag{7}$$

The numerical values for the constants are: $a = 0.8$ atm.; $\alpha_1 = 0.0202$ cc./cc./atm.; $\alpha = 0.0200$ cc./cc./atm.; $V = 1$ cc./min. Q is directly proportional to the diffusion constant which for N_2 is about half that of oxygen. Q is therefore for nitrogen 65 cc./cm./cm.^{2}/min./atm.

If we have a 1% decrease in solubility at the exit of the *rete* the equilibrium pressure P would be 26 atmospheres. Theoretically, therefore, one could get enough pressure built up. It is obvious that the rate of N_2 deposition by this means would be exceedingly slow, since each cubic centimeter of blood would only transport in 0.16 mm.^{3} N_2/min.

A striking thing about the nitrogen tension in the swimbladders of our marine fishes is that in most of them it increases linearly with the depth, as if the nitrogen were produced as a constant percentage of the oxygen secretion. This observed phenomenon ($P_{N_2}/P_{O_2} = 2$–15%, and independent of pressure) could only be derived from the equations 5 and 7 if R were either proportional to the pressure or if it were constant, neither of which assumption seems likely to be true. There is a further difficulty with the proposed theory where an x-substance produces the lowering of the solubility coefficient, in that if the substance were diffusible it would

be likely to concentrate enormously in the inner part of the *rete*. And if it were not diffusible how would it have entered the blood stream in the first place? Another serious difficulty is that physostomes like the whitefish evidently lack a typical *rete*.

Conclusions. The nitrogen-to-argon ratio in the swimbladder of deep sea fishes was found to be approximately that of the sea water and atmosphere, confirming what was found earlier by Schloesing and Richard (1896). In these fishes, therefore, the nitrogen has not been chemically secreted.

The theory of Koch (1934) that nitrogen and argon might be secreted into the swimbladder by a decreased solubility coefficient in the effluent blood of the *rete* has been evaluated quantitatively. Although high enough equilibration pressures of nitrogen could be achieved by a small change in the solubility coefficient, the constant ratio N_2/O_2 at all depths, as actually found, cannot readily be explained by this theory. The theory may not apply to physostomes, like the whitefish, which seem to lack a typical *rete*.

During this work I have received many suggestions from stimulating discussions with Dr. A. C. Redfield and Dr. L. van Dam at the Woods Hole Oceanographic Institution. I am much obliged to Dr. A. O. C. Nier and to Mr. Bailey Donnally at the Physics Department of the University of Minnesota for mass spectrographic analyses of the argon/nitrogen ratio in the swimbladder gases. Dr. George Wislocki, Department of Anatomy, Harvard Medical School, has generously given his help and advice with regard to the histological material. This long-preserved material was most skillfully prepared by Mrs. Edith Herman and photographed by Mr. Leo Talbert of the same department. I am much indebted to Dr. C. M. Breder, Jr., of the American Museum of Natural History, for arranging our work at the Lerner Marine Laboratory, and I wish to thank Mr. Michael Lerner and the staff of the Lerner Marine Laboratory for all the help they extended to us. Mr. W. C. Schroeder of the Woods Hole Oceanographic Institution gave us the best of facilities on board the dragger *Cap'n Bill II*, and for much assistance in the laboratory and with the manuscript thanks are due to Dr. L. van Dam and Mrs. Susan I. Scholander.

Summary

1. The structure and dimensions are given for the *rete mirabile*. It is interpreted as a counter current diffusion exchange mechanism between the afferent and efferent *rete*.

2. This diffusion exchange has been quantitatively evaluated for O_2, CO_2, and N_2. The arrangement makes possible the maintenance of a steep tension gradient within the *rete*, so that the oxygen loss from the leaving blood can be extremely low.

3. Quantitative evaluation of the efficiency of the *rete* diffusion makes it clear that the limiting equilibrium pressure in the swimbladder could be extremely high, and that the limiting factor lies mainly in the dissociation pressure of the chemical or physical reaction which ultimately splits off the oxygen.

4. The nature of the responsible compound and reaction is unknown. The anatomical arrangement of the glandular structure and the *rete* is suggestive of a

cellular secretion of oxygen rather than of a mechanism that splits off oxygen in the blood.

5. Data on the deposition of nitrogen against high pressures which were previously found in deep sea fish have been confirmed and extended. The argon-to-nitrogen ratio in the swimbladder gas suggests that the nitrogen has entered the swimbladder via some physical mechanism. The possibility that this is brought about by a lowering of the nitrogen solubility coefficient in the efferent *rete* is discussed.

LITERATURE CITED

AKITA, Y. K., 1936. Studies on the physiology of the swimbladder. *J. Fac. Sci., Tokyo, Sect. IV, Zool.*, **4**: 111–135.

BAZETT, H. C., L. LOVE, M. NEWTON, L. EISENBERG, R. DAY AND R. FORSTER II, 1948. Temperature changes in blood flowing in arteries and veins in man. *J. Applied Physiol.*, **1**: 3–19.

BOHR, C., 1905. Blutgase und respiratorischer Gaswechsel. *In* W. Nagel, *Handbuch der Physiologie des Menschen*, **1**: 54–222. Friedrich Vieweg und Sohn, Braunschweig.

COPELAND, D. E., 1952. The histophysiology of the teleostean physoclistous swimbladder. *J. Cell. Comp. Physiol.*, **40**: 317–334.

FÄNGE, R., 1953. The mechanisms of gas transport in the euphysoclist swimbladder. Acta Physiol. Scandinavica, vol. 30, suppl. 110, 133 pp.

HALDANE, J. S., 1922. Respiration. Yale Univ. Press, New Haven, Conn.

HALL, F. G., 1924. The functions of the swimbladder of fishes. *Biol. Bull.*, **47**: 79–126.

HARGITAY, B., AND W. KUHN, 1951. Das Multiplikationsprinzip als Grundlage der Harnkonzentrierung in der Niere. *Zeitschr. Elektrochem. Angewandte Physik. Chem.*, **55**: 539–558.

HARVEY, E. N., D. K. BARNES, W. D. MCELROY, A. H. WHITELEY, D. C. PEASE AND K. W. COOPER, 1944. Bubble formation in animals. I. Physical factors. *J. Cell. Comp. Physiol.*, **24**: 1–22.

HÜFNER, G., 1892. Zur physikalischen Chemie der Schwimmblasengase. *Arch. Anat. Physiol., Physiol. Abth.*, 54–80.

JACOBS, W., 1930. Untersuchungen zur Physiologie der Schwimmblase der Fische. I. Über die "Gassekretion" in der Schwimmblase von Physoklisten. *Zeitschr. Vergleichende Physiol.*, **11**: 565–629.

JACOBS, W., 1934. Untersuchungen zur Physiologie der Schwimmblase der Fische. III. Luftschlucken und Gassekretion bei Physostomen. *Zeitschr. Vergleichende Physiol.*, **20**: 674–698.

KOCH, H., 1934. L'émission de gaz dans le vésicule gazeuse des poissons. *Rev. Questions Sci., Ser. 4*, **26**: 385–409.

KROGH, A., 1919. The rate of diffusion of gases through animal tissues, with some remarks on the coefficient of invasion. *J. Physiol.*, **52**: 391–408.

LEDEBUR, J. VON, 1937. Über die Sekretion und Resorption von Gasen in der Fischschwimmblase. *Biol. Rev.*, **12**: 217–244.

LEINER, M., 1938. Die Physiologie der Fischatmung. Akad. Verlagsgesellsch. M.B.H., Leipzig.

LEINER, M., 1940. Das Atmungsferment Kohlensäureanhydrase im Tierkörper. *Naturwissenschaften*, **28**: 165–171.

MÜLLER, J., 1840. Ueber Nebenkiemen und Wundernetze. *Arch. Anat. Physiol. Wissen schaftl. Med.*, 101–142.

PAPPENHEIMER, J. R., E. M. RENKIN AND L. M. BORRERO, 1951. Filtration, diffusion and molecular sieving through peripheral capillary membranes. *Amer. J. Physiol.*, **167**: 13–46.

POWERS, E. B., F. G. HOPKINS, T. A. HICKMAN AND L. M. SHIPE, 1932. The relation of respiration of fishes to environment. *Ecol. Monogr.*, **2**: 385–473.

ROSTORFER, H. H., 1942. The gas content of the swimbladder of the rock bass, *Ambloplites rupestris*, in relation to hydrostatic pressure. *Biol. Bull.*, **82**: 138–153.

SAUNDERS, R. L., 1953. The swimbladder gas content of some freshwater fish with particular reference to the physostomes. *Canadian J. Zool.*, **31**: 547–560.

SCHOLANDER, P. F., AND L. VAN DAM, 1953. Composition of the swimbladder gas in deep sea fishes. *Biol. Bull.*, **104**: 75–86.

SCHOLANDER, P. F., AND L. VAN DAM, 1954. Secretion of gases against high pressures in the swimbladder of deep sea fishes. I. Oxygen dissociation in blood. *Biol. Bull.*, **107**: 247–259.

SCHOLANDER, P. F., S. C. FLEMISTER AND L. IRVING, 1947. Microgasometric estimation of the blood gases. V. Combined carbon dioxide and oxygen. *J. Biol. Chem.*, **169**: 173–181.

SCHLOESING, T., AND J. RICHARD, 1896. Recherche de l'argon dans les gaz de la vessie natatoire des poissons et des physalies. *C. R. Acad. Sci., Paris*, **122**: 615–617.

STRITTMATTER, C. F., E. G. BALL AND O. COOPER, 1952. Glycolytic activity in the swimbladder gland. *Biol. Bull.*, **103**: 317.

WOODLAND, W. N. F., 1911. On the structure and function of the gas glands and *retia mirabilia* associated with the gas bladder of some teleostean fishes, with notes on the teleost pancreas. *Proc. Zool. Soc. London*, 183–248.

THE EFFECTS OF LIGHT ON TEMPERATURE SELECTION IN SPECKLED TROUT SALVELINUS FONTINALIS (MITCHILL) [1]

CHARLOTTE M. SULLIVAN AND KENNETH C. FISHER

University of Toronto, Toronto 5, Canada

It will be generally accepted that the locomotory behavior of any animal in its ordinary life is a reaction or response resulting from the host of stimuli both internal and external which are continually acting upon it. The physiological analysis of such resultants is so obviously complex that here, as in most other fields, investigators have felt it desirable to commence investigation in simplified situations where the number of independent variables operating simultaneously is kept as few as possible. As a consequence much has been learned about the reactions of a great variety of animals to light, when temperature, gravity, humidity, nutritional state, etc., are kept constant; of the reaction to temperature if light and the other possible variables are kept constant, and so on. The relatively enormous literature which has resulted has been admirably summarized by Fraenkel and Gunn (1940).

It is evident, however, that if a complete understanding is to be obtained of the sensori-motor mechanisms and the nervous system which integrates them, experiments will be required in which the reactions to the simultaneous application of several stimuli are studied. Initial steps in this direction have already been made in a number of instances. Even Mendelssohn (1902) in his early work with protozoa showed that the responses of the organisms to two simultaneously acting stimuli were varied and depended upon the experimental arrangements. With the proper conditions the stimuli reinforced one another. Under other conditions the effect of one or the other stimulus prevailed—to a degree which was related to the relative strengths of the two stimulating conditions.

Of the stimuli which can be combined, light and temperature are among the most easily controlled from an experimental standpoint and are possibly among the most important factors affecting organisms from moment to moment. It is well known that when organisms are free to move about in a gradient of temperature they tend to be confined to some particular relatively small range of temperature which they are then said to have "selected." (See Fraenkel and Gunn, 1940; Sullivan and Fisher, 1953; Stinson and Fisher, 1953, for references.) It is equally well established that given both a lighted and a darkened area many organisms select one or the other (Fraenkel and Gunn, 1940; for fish specifically see Schiche, 1921; Young, 1935; Breder and Rasquin, 1950; Collins, 1952 and Lowe, 1952). There are instances in which some particular intensity was selected in a gradient of light intensity (Weber, 1929; Henschel, 1929; Totze, 1933; Ullyott, 1936 and Herrström, 1949).

[1] This investigation was assisted by a grant from the National Research Council of Canada.

The interrelations of the effects of light and temperature are clearly of interest. Some have already been described. Thus Herter (1923b) reported that house crickets selected a higher temperature in diffuse daylight than they did when exposed to lower levels of illumination, while red ants (Herter, 1923b, 1924) and flower beetles (Bodenheimer and Schenkin, 1928) selected a lower temperature in daylight than when less intensely illuminated. Actually there are apparently some instances in which no interrelation exists. Thus Herter (1923a, 1924) found that the temperatures selected by fireflies and field crickets, respectively, were independent of the incident light intensity.

The distribution of organisms in such a non-uniform environment as a temperature gradient is characterized not only by its mode, which is taken as the condition selected, but also by its spread. The latter indicates the sharpness or precision of the selection and has been measured by the standard deviation of the distribution. Herter (1923b, 1924) showed in this way that the precision of temperature selection by adults of *Acheta domestica* and *Lyogryllus campestris*, respectively, was greater (*i.e.*, the standard deviation was smaller) in light than in the dark. Although Bodenheimer and Schenkin (1928) do not discuss this point, the standard deviations of the distributions they observed for the flour beetle and for *Scanthius aegyptius* were not the same in darkness and in light. In these cases temperature selection appeared more precise in the dark than in the light.

The effect of the ambient light intensity on temperature selection by fish does not appear to have been examined, although the reverse experiment, the effect of different constant temperatures on light selection has been reported (*cf.* Andrews, 1946 and the preliminary work by Collins, 1952). Moreover, although the behavior of fish in response to simultaneously operating factors has been examined in certain connections, the distribution of fish in an environment in which there were non-uniformities of light as well as of temperature does not appear to have been studied. We have therefore determined the selected temperature of trout and the precision of selection at two light intensities and, because it was a necessary prerequisite, the precision of selection in successive experiments with the same fish; data were also obtained which establish the nature of the distribution to be expected in an environment in which non-uniformities of light and temperature are superimposed.

Materials and Methods

The experimental animals were young speckled trout, *Salvelinus fontinalis* Mitchill, two to three inches long. They were obtained from a private hatchery [2] and held in running Toronto tap water.

For all the experiments to be discussed, a five-foot long copper-bottomed trough with glass sides was used. This apparatus, the method of establishing and maintaining a temperature gradient in it and the procedure followed to determine the temperature selected by the experimental organisms have been described previously (Sullivan and Fisher, 1953).

For the experiments to be reported here the trough was provided with an arrangement of lights and opaque light shields ("covers") by means of which the desired lighting conditions were set up. A diagram showing the trough and the

[2] It is a pleasure to acknowledge the cooperation of Mr. Herbert Pearen of Hornings Mills, Ontario, in this connection.

light shields in cross section will be found in Figure 1. The trough in which the fish were placed is designated as (a). Shield (b) was closely applied to the back of the trough (a), and effectively prevented any entrance of light from that direction. Shield (c) similarly covered the front of the trough except for a longitudinal slit along the bottom which permitted observation of the fish in the trough. Shield (d) was made of several overlapping sections so that by removing appropriate sections and adjusting the remaining ones any desired region of the trough could be illuminated. To make the boundary between the lighted regions and the dark

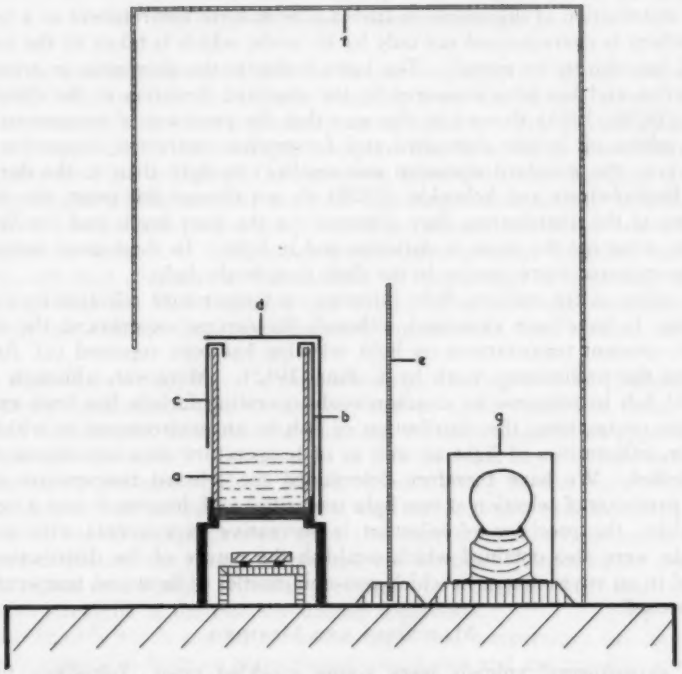


FIGURE 1. Diagram of trough and light shields; cross-section.

regions as sharp as possible, vertical shields were hung from the edges of cover (d) to within ½ inch of the surface of the water in the trough.

Light was provided by a row of six electric bulbs evenly spaced along the back of the apparatus. The illuminated surfaces of shields (e) and (f) were left their natural color (light buff) so as to provide diffuse reflection of light into the uncovered regions of the trough. All other surfaces were painted flat black.

Two types of experiments were done. For the first type, in which the whole trough was evenly illuminated, all the sections of shield (d) were removed. Under these circumstances "bright" light was provided by using 25-watt bulbs in the six sockets; "dim" light was obtained by using 7½-watt bulbs and covering the whole row of bulbs with thin white cardboard ((g) in Fig. 1).

For the second type of experiment, in which only part of the trough was illuminated, the required region was uncovered by adjusting shield (d). Six 25-watt bulbs were used. The intensity of light in the uncovered region was reduced as required by placing one or more layers of white paper over the opening in shield (d).

Results

The effect of the incident light intensity on the selection of temperature may be determined either by studying a given group of fish at each of the light intensities to be used, or by using several groups of fish each of which is studied at only one intensity. The first of these procedures involves the subjection of a group of fish to two or more light intensities in succession. In it there is clearly a possibility that the behavior in the second and any subsequent exposures in the gradient may be affected by the previous experience in the gradient. A serial effect of this kind is of interest, of course, not only because it might affect the interpretation of the data obtained by procedure one, but also for its own sake, since the existence of any such serial effect would be indicative of a kind of learning by the organisms. The first experiments to be discussed here provide data about the serial effect in gradient experiments.

Effect of successive exposures to the temperature gradient

Each of nine groups of fish was studied upon three or more occasions, these being separated by one week or more. The light intensity used ("bright," see Methods) was constant along the gradient and was the same throughout all of these experiments. Typically the histogram which described the distribution of the fish in each experiment was definitely peaked. There was a considerable variation in the number of observations in the modal group from day to day and from group to group of experimental animals. It was not possible, however, to detect any consistent change in the value of the temperature selected from trial to trial other than those that normally occur with changing seasons (Sullivan and Fisher, 1953).

It seems then that as far as the actual temperature selected was concerned there was no serial effect. The sharpness of the selection on the other hand, as indicated by the standard deviations of the various distributions, did appear to change with successive determinations. These standard deviations are listed in Table I and it is evident from the mean values that the distributions tended to be spread out more on the temperature axis in the initial trial than in subsequent ones. Moreover the tendency for the standard deviation to decrease in successive trials may be seen in each of the individual groups of fish with the exception of groups 4 and 6—and it is particularly apparent in the cases of groups 1, 2 and 3, which were each observed in four successive experiments. The mean value for the nine groups in trial 1 is significantly different, in the statistical sense, from the mean value in trial 2 ($t = 2.5$, $n = 8$, probability 4%). The mean value for the groups in trial 1 is likewise different from that in trial 3 ($t = 2.6$, $n = 8$, probability 4%).

In these experiments, then, the standard deviation of the distribution of a group of fish in the gradient decreased somewhat with successive tests up to the third or fourth such tests. It is not at all apparent how this could have been an artifact resulting from the experimental conditions and so it must be concluded that it was a property of the organisms themselves. Experience in the gradient *per se* or be-

TABLE I

Standard deviations of selection histograms obtained in successive trials of nine groups of fish in temperature gradients

Group	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
1	4.38	3.48	3.53	3.02	3.02
2	4.95	5.07	4.71	4.32	
3	4.56	4.08	3.96	3.60	
4	2.94	2.91	3.09		
5	3.44	3.36	2.84		
6	4.23	4.23	4.17		
7	4.62	3.84	4.38		
8	4.62	3.99	3.75		
9	4.68	3.24	3.02		
Mean	4.27	3.80	3.72	3.68	

cause of the development in the fish of a familiarity with the apparatus led to a sharper, *i.e.*, more precise, selection of temperature.

Effect of incident light intensity on temperature selection

The experiments made to determine the precision of temperature selection in bright and dim light involved four different groups of fish, each consisting of ten individuals. Each group was divided into two lots, designated (a) and (b), respectively. Lot (a) of each group was examined first in dim light and then, in a second experiment, in bright light, while lot (b) was examined first in bright

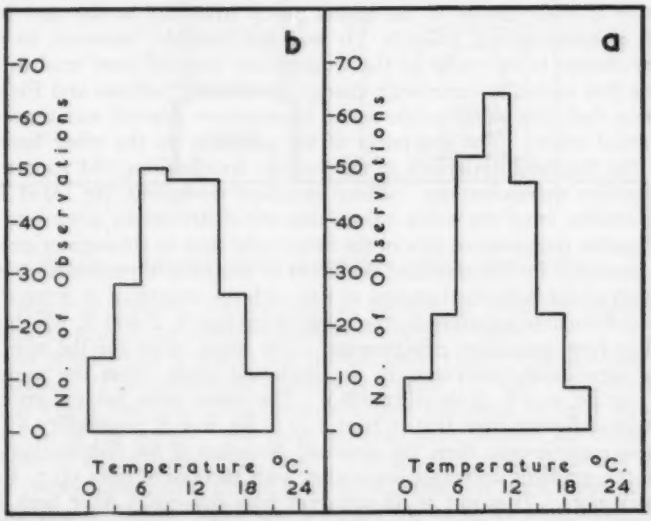


FIGURE 2. Distribution of trout in the temperature gradient. a) gradient brightly lighted; b) gradient dimly lighted.

light and then, in a second experiment, in dim light. These two determinations of the selected temperature were the only ones made on each of the two lots of groups one, two and three. A third determination was made, however, in the case of the two lots of group four, and for this determination each lot was exposed again to the initial illumination condition which it had experienced.

Histograms showing the average distribution of the organisms in dim and bright light respectively are shown in Figure 2a and b. It may be seen that a more peaked distribution occurred in the dim light (Fig. 2a). The standard deviation of the distribution in Figure 2a is 4.08 as compared with 4.62 in Figure 2b. Apparently the precision of selection was greater in the dim than in bright light. Such experiments as these which have just been discussed involved subjection to two different light intensities successively, and, as was noted above, repetition of temperature selection experiments tends to elicit sharper selection. As a consequence it could be expected that the apparent difference between the sharpness of

TABLE II

Group	Dark	Light	Time in gradient
1	(a) 4.11 →	(b) 5.46	In this series Ia and Ib were tested
	3.87 ←	4.98	one day and five days later Ia was tested twice, first in light, then in dark
2	(a) 3.46 →	(b) 4.86	First
	3.81 ←	4.23	Second
3	(a) 4.71 →	(b) 4.83	First
	4.02 ←	4.20	Second
4	(a) 4.35 →	(b) 4.59	First
	3.96 ←	4.56	Second
	3.72 →	3.96	Third

selection in dim and bright light would be greater when the first determination was made in bright light than when it was made in dim light. In the one case, the effect of repetition would reinforce the change due to the difference in light intensity, while in the other, it would tend to cancel it. It is of interest, therefore, to examine the standard deviations of the individual distributions which were combined to produce Figure 2. They are given in Table II. The values joined by arrows were determined on the same lot of fish, the sequence of the experiments in light and dark being indicated by the direction of the arrows.

If one examines the instances in which the determination was made first in bright and then in the dim illumination (one instance for each of groups 2 and 3 and two instances for group 4) it may be seen that without exception the standard deviations are considerably greater in bright than in dim light. When, however, the instances are taken in which a given group of fish was observed first in dim and then in bright light, the standard deviation in the second experiment is greater than that in the first in only one instance (Group 3a). In one case (4a, second and third trials) the standard deviations are the same while in the remaining three

cases the standard deviation of the first determination in dim light is actually considerably less than that of the second experiment in bright light.

As was to be expected then, the serial effect enhanced, if anything, the change in standard deviation seen when the organisms were examined first in bright light but it reduced or reversed the change due to lighting conditions when the experiments in dim light were done first.

There is then no question that a given lot of fish select more sharply in dim light than in bright light and it is evident that the serial effect operates even though the additional factor of light intensity is also involved.

These experiments in which the standard deviation of the temperature selection distribution were determined at the different light intensities also provide information about the temperature selected at the two light intensities. Nine comparisons of the temperature selected by the (a) lots at one light intensity with the temperature selected by the (b) lots at the other intensity may be made.

The pairs of values, in ° C., were as follows, the top member of each pair being the observations in bright light in each case:

13.2	13.2	11.5	8.1	12.2	10.3	10.5	7.5	7.8
13.4	13.4	10.9	8.1	10.5	10.6	10.5	7.9	9.0

The mean value at both intensities was 10.5° C. It may be concluded that the temperature selected by brook trout is not dependent on the ambient light intensity within the limits of intensity used here. The selected temperatures listed for both bright and dim light do show a trend toward lower values, because the determinations were made in the autumn when a seasonal shift in the temperature selected by trout regularly occurs (Sullivan and Fisher, 1953).

Interference between selection of light and temperature

For this part of the study two experiments, *i.e.*, determinations of the distribution of five organisms in the experimental temperature gradient, were done each day. In the first or control experiment a group of five fish was observed for two hours (five fish, each located forty-eight times, making a total of two hundred and forty observations) in the temperature gradient with the gradient trough completely covered. In this experiment the selected region was established. In the second experiment a comparable group of organisms from the same laboratory stock was used, and the region selected in the control experiment was exposed to the desired illumination. The highest intensity employed will be arbitrarily designated 100% (this corresponds to an incident illumination density of approximately 15 foot candles); other intensities will be specified relative to this. It may be recorded here that as a consequence of the particular light arrangements used in these experiments (described under Methods) the intensity of light in the darkened regions of the trough was found to be practically independent of the intensity in the lighted region. The actual intensity of the darkened parts, as well as the intensity of the whole trough when it was uniformly lighted with "dim" light was approximately 1% on the arbitrary scale.

Typical examples of the distributions found with the selected region (always taken to be one third of the length of the gradient trough) illuminated at the

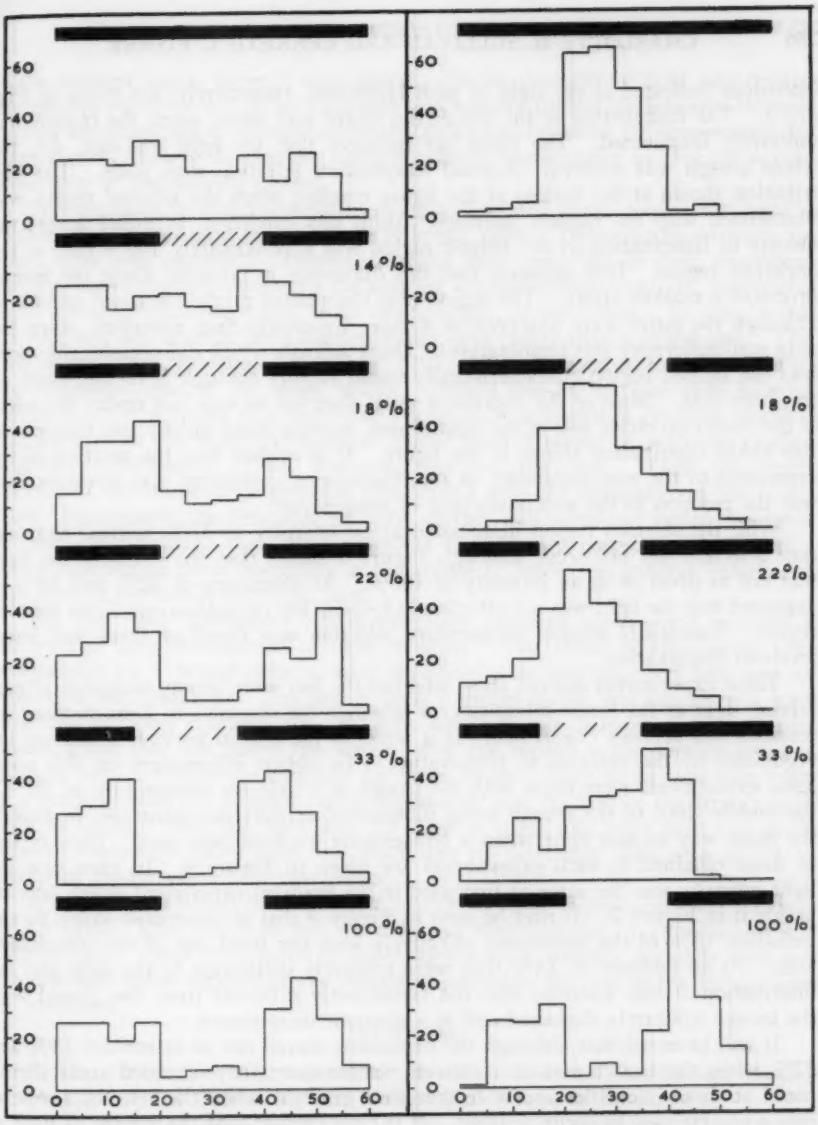


FIGURE 4. FIGURE 3.

FIGURE 3. Distribution of trout in the temperature gradient. The horizontal axis represents inches along the trough and the vertical axis represents frequency of observations of fish. Light conditions in each experiment are indicated by the black bars. Illumination of a particular region of the trough is indicated by interruption of the black bar over that region. The relative intensity of illumination of the exposed region in each experiment is given by the figure at the upper right of each distribution.

FIGURE 4. Distribution of trout in the trough at constant temperature. For further explanation see the legend of Figure 3.

intensities indicated at the right of each histogram, respectively, are given in Figure 3. The distribution at the top of the figure was found when the trough was uniformly illuminated. The black bar indicates that the light was dim, *i.e.*, the whole trough was covered. Normal temperature selection took place. The distribution shown at the bottom of the figure resulted when the selected region was illuminated with the highest intensity. With this condition, as noted above, the density of illumination in the lighted region was approximately 100 × that of the darkened region. It is apparent that this difference in intensity along the trough produced a marked effect. The region now illuminated rarely contained organisms although the latter were observed in it more frequently than elsewhere when the only non-uniformity was temperature. Those animals which did occasionally move into the lighted region characteristically swam rapidly through it to the shade on the other side. Most of the organisms were observed to stay just under the edges of the covers on either side of the lighted area, moving about quietly and thus giving rise to the distribution shown in the figure. It is evident that the reaction of the organisms to the non-uniformity of the illumination conditions was stronger than was the reaction to the non-uniformity of temperature.

With the selected region illuminated at an intensity of 33%, normal temperature selection did not occur although Figure 3 shows that the effect of the light was not as great as at an intensity of 100%. At intensities of 22% and 18%, it appeared that the light was not effective in keeping the organisms out of the selected region. Essentially normal temperature selection was found at these and lower levels of illumination.

These experiments did not show whether the fish were simply incapable of perceiving light at the lower intensities, or whether the response to light at these intensities was actually "overruled," as it were, in the central nervous system of the organisms by the reaction to temperature. To obtain information on this point some experiments were made with the trough at a uniform temperature of 11° C., the middle third of the trough being illuminated at different intensities in exactly the same way as was done when a temperature gradient was used. Data typical of those obtained in such experiments are given in Figure 4. In each case the light intensity was the same as that used in the gradient experiment which appears beside it in Figure 3. It may be seen in Figure 4 that all intensities down to and including 18% of the maximum, effectively kept the trout out of the illuminated area. At an intensity of 14% they were relatively indifferent to the light and the distribution at this intensity was not significantly different from that found with the trough uniformly darkened and at a constant temperature.

It will be noted that although the organisms stayed out of intensities 18% and 22% when the trough was at a uniform temperature, they occupied areas illuminated at these intensities in the temperature gradient when the selected temperature was arranged to occur in them. It is thus evident that the failure of light at the 18 and 22% levels to modify the distribution in the temperature gradient was not the consequence of an inability to perceive or appreciate such intensities. Instead it must be concluded that there was competition between the responses to light and to temperature in the gradient. The intensity of light which the fish would enter in the presence of a temperature gradient was considerably greater than the intensity which they would enter if no gradient were present. At high light intensities the negative response to light predominated and the trout were kept out

of the selected region when it was lighted. As the intensity of light was reduced the relative effectiveness of the light and temperature stimuli on behavior changed, and at an illumination of approximately 20%, the response to temperature had come to predominate so that the animals selected temperature as though no non-uniformity of illumination existed.

The reactions to non-uniformities of light described above were interpreted as "selections" of dim light in preference to bright light. Theoretically, at least, this could have been incorrect. Instead, it might have been that in their movements the organisms tended to preserve the existing condition, dim or bright, rather than move across a demarcation line, from dim to bright, or vice versa. In other words, the fish may not actually have selected a dim light rather than a bright one. They may simply have "hesitated," as Breder (1951–52) expressed it, to move from the one condition, dim, to the other, bright. Should this be the case then it would follow that the selection of a particular region in a temperature gradient would be *less* sharp with the region in question shaded than it would be with the gradient uniformly illuminated. This, however, was not the case. When the region selected in the temperature gradient was shaded with the remainder of the trough highly illuminated, the fish selected very sharply—all remained under the cover. It must be concluded that these reactions to light are correctly interpreted as active selection of some intensities in preference to others; in general, selection of dim light in preference to bright light.

The fish used throughout the investigation reported above were exposed to the normal daily fluctuations of light intensity. The effect of eliminating these fluctuations for a period prior to such experiments as are described here was examined in two instances. For one of these a group of fish was placed in a white tank and exposed continuously for three weeks to the light of a 60-watt lamp placed about two feet above the water. For the second a comparable group of fish was kept in continuous darkness for three weeks. Each group was then tested in a gradient with the selected region illuminated and the rest of the trough darkened as usual. Both the light and the dark adapted fish were kept out of the selected region by 100% light and there was no detectable difference in the behavior or distribution of the two groups.

It would be expected that the effect of light in these experiments was mediated by the eyes. That this is in fact the case was demonstrated by experiments with blinded fish. These were found to select temperature perfectly normally even with the selected region exposed to the maximum intensity, the rest of the trough being in "darkness."

Summary

1. The distribution of groups of five speckled trout in a longitudinal gradient of temperature was studied under a variety of conditions of illumination.

2. The modes of the distributions, *i.e.*, the selected temperatures, did not change with successive determinations separated by a week or more. However the selection of temperature became more precise as the fish acquired experience in the apparatus.

3. Selection of temperature was apparently more precise at low light intensities than at high intensities although the actual temperature selected did not vary with the light intensity.

4. Some experiments were done in which only part of the experimental environment was illuminated. When temperature was constant trout would not remain in the lighted part unless the intensity of illumination was very low. When, in a gradient of temperature, the illuminated part was made to coincide with the region normally selected by virtue of the temperature, it was found that at high light intensities the organisms did not appear in the illuminated region. Clearly a response to light prevailed. At intermediate and low light intensities, on the other hand, it was the response to temperature which prevailed and in spite of the illumination the organisms selected temperature in a normal way.

LITERATURE CITED

ANDREWS, C. W., 1946. Effect of heat on light behaviour of fish. *Trans. Roy. Soc. Canada*, **40**: Sect. V, 27–31.

BODENHEIMER, F. S., AND D. SCHENKIN, 1928. Über die Temperaturabhängigkeiten von Insekten. I. Über die Vorzugstemperatur einiger Insekten. *Zeitschr. Vergl. Physiol.*, **8**: 1–15.

BREDER, C. M., JR., 1951–52. Studies on the structure of the fish school. *Bull. Amer. Mus. Nat. Hist.*, **98**: 1–27.

BREDER, C. M., JR., AND P. RASQUIN, 1950. A preliminary report on the role of the pineal organ in the control of pigment cells and light reactions in recent teleost fishes. *Science*, **111**: 10–12.

COLLINS, G. B., 1952. Factors influencing the orientation of migrating anadromous fishes. *Fishery Bulletin of the Fish and Wildlife Service*, **52**: Bull. No. 73, 374–396.

FRAENKEL, G. S., AND D. L. GUNN, 1940. The orientation of animals. Oxford, Clarendon Press.

HENSCHEL, J., 1929. Reizphysiologische Untersuchungen an der Kasemilbe, *Tyrolichus casei* (Oudemans). *Zeitschr. Vergl. Physiol.*, **9**: 802–837.

HERRSTRÖM, G., 1949. Illumination preferendum and adaptation experiments with *Agonum dorsale* Pont. (Col., Carabidae). *Oikos*, **1**: 48–55.

HERTER, K., 1923a. Untersuchungen über den Temperatursinn der Feuerwanze (*Pyrrhocoris apterus* L.). *Biol. Zentralbl.*, **43**: 27–30.

HERTER, K., 1923b. Untersuchungen über den Temperatursinn der Hausegrille (*Acheta domestica* L.) und der roten Waldameise (*Formica rufa* L.). *Biol. Zentralbl.*, **43**: 282–285.

HERTER, K., 1924. Untersuchungen über den temperatursinn einiger Insekten. *Zeitschr. Vergl. Physiol.*, **1**: 221–228.

LOWE, ROSEMARY H., 1952. The influence of light and other factors on the seaward migration of the silver eel (*Anguilla anguilla* L.). *J. An. Ecology*, **21**: 275–309.

MENDELSSOHN, M. M., 1902. Recherches sur l'interference de la thermotaxie avec d'autres tactismes et sur le mecanisme du movement thermotactique. *J. de Physiol. et de Pathol. Generale*, **4**: 475–496.

SCHICHE, O. E., 1921. Reflex biologische an *Amiurus nebulosus*. *Zool. Jahrb. Jena*, **38**: 49–112.

STINSON, R. H., AND K. C. FISHER, 1953. Temperature selection in deer mice. *Canad. J. Zool.*, **31**: 404–416.

SULLIVAN, C. M., AND K. C. FISHER, 1953. Seasonal fluctuations in the selected temperature of speckled trout, *Salvelinus fontinalis* (Mitchill). *J. Fish. Res. Bd. Can.*, **10**: 187–195.

TOTZE, R., 1933. Beitrage zur Sinnesphysiologie der Zecken. *Zeitschr. Vergl. Physiol.*, **19**: 110–161.

ULLYOTT, P., 1936. The behaviour of *Dendrocoelom lacteum*. II. Responses in non-directional gradients. *J. Exp. Biol.*, **13**: 265–278.

WEBER, H., 1929. Biologische Untersuchungen an der Schweinelaus (*Haematopinus suis* L.) unter besonderer Berücksichtigung der Sinnesphysiologie. *Zeitschr. Vergl. Physiol.*, **3**: 389–474.

YOUNG, J. Z., 1935. The photoreceptors of lampreys. I. Light sensitive fibers in the lateral line nerves. *J. Exp. Biol.*, **12**: 229–238.

ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1954

ABSTRACTS OF SEMINAR PAPERS

JULY 6, 1954

An action spectrum analysis of the early development of Drosophila. ALLEN S. GOLDMAN.

An action spectrum analysis was made in order to determine the identity, location and role of photo-inactivated compounds in the early development of Drosophila. The superficial cleavage of this egg presents two early stages in which radiation damage to the nucleus can be differentiated from radiation damage to the cytoplasm. Eggs radiated with small doses of ultraviolet when the nuclei are exposed to U.V. radiation either develop normally or show an abnormal retraction of their protoplasm resulting in complete early inhibition of their development.

Eggs with cytoplasm exposed and nuclei protected within the interior of the egg were radiated with larger doses of several monochromatic wave-lengths of the U.V. spectrum. These embryos either developed normally or showed evisceration of their yolk or herniation of their intestines. Differentiation of the rest of the embryos is apparently complete. Eggs radiated with U.V. wave-lengths from 2700–3131 Å show some nuclear damage because of the deeper penetration of the longer wave-lengths through the transparent cytoplasm. A mathematical confirmation provides evidence that the radiation damage to the cytoplasm can be differentiated from radiation damage to the nuclei.

By means of an action spectrum analysis, a quantitative assay of the spectral efficiencies in causing the abnormalities characteristic of eggs radiated with cytoplasm exposed indicates that the absorbing compounds affected are purine- and pyrimidine-containing compounds. Furthermore, since the affected purine- and pyrimidine-containing compounds must be located in the cytoplasm, a working hypothesis is adopted that cytoplasmic nucleic acid, RNA, is the affected compound and that damage to this compound leads to the observed abnormal tissue movements and presumably to abnormal tissue interactions.

Radiosensitivity with respect to the estrus cycle in the mouse. R. RUGH AND H. CLUGSTON.

Radiobiological studies with the mouse indicate that reproducible results can be achieved better by using males rather than females. This fact led to a study of survival of female CF_2 mice receiving male LD 50/30-day x-ray exposures but at different phases of the estrus cycle.

Under identical geometrical conditions of x-irradiation, litter-mate male mice exhibited a lower survival value than any females, no matter in what stage of estrus they were x-irradiated. Thus, the male CF_1 mouse is considered to be more radiosensitive than the female.

The females x-irradiated during estrus had the best survival value (66% in one experiment) as contrasted with those x-irradiated during diestrus (36% survival at 625 r). The intermediate stages of pre- and post-estrus are of short duration (6 hours) and are not easily distinguished by the vaginal smear method. These stages are critically defined as having approximately equal numbers of cornified epithelial cells and leucocytes. They were therefore considered together. In the three sets of data, involving 500 mice, the survival of these two

combined stages was intermediate between the estrus and diestrus in one experiment and tended to be comparable to estrus survival in the others. This is explained on the probability that during pre-estrus there is a greater secretion of estrogenic hormones, and the proportion of pre- and post-estrus mice in the three experiments would vary.

While there is a correlation in total body weight of the female mouse with the phases of estrus, suggesting hydration changes with maxima during diestrus, this is not considered as the primary factor attributing to the female cyclic changes in radiosensitivity. Rather, periods of greatest radiosensitivity are correlated with estrus, and it is believed that the elaboration of estrogens provide a chemical source of protection. This concept is supported by experimental data in protection studies.

The relationship between the surface structure and the electrophoretic mobility of E. coli bacteria. CHARLES C. BRINTON, JR., ANNE BUZZELL AND MAX A. LAUFFER.

Microscope electrophoresis studies revealed that a variant of *E. coli* B bacteria showed either organisms with a mobility of approximately 2 microns/sec./volt/cm., or organisms with a mobility of approximately 3.6 microns/sec./volt/cm. in a medium with ionic strength .018 buffered to pH 7.0 with phosphate salts. Intermediate values of mobility were not observed, but the relative numbers of fast and slow organisms varied from culture to culture. Bacteria selected for resistance to T_1 bacteriophage were found to be uniformly fast.

In seeking an explanation for the difference between fast and slow organisms, it occurred to us that a difference in the friction coefficient of the particle might be responsible. The intrinsic viscosities of a concentrated suspension of fast organisms and of a concentrated suspension of slow organisms were measured. The value for the slow organisms was found to be 3.3 times as great as that of the fast. It was reasoned that such a high intrinsic viscosity could be brought about by surface irregularities. Electron microscopy confirmed this by revealing on the slow organisms filaments about 150 Å in diameter and of length up to a micron or more, while the fast organisms were without such filaments. The filaments could be removed by agitating with a Waring Blendor; previously slow organisms became electrophoretically fast. De-filamented organisms were viable, and, upon incubation and multiplication, filaments were produced and the organisms again became slow.

The difference between filamentation and non-filamentation may be related to the difference between rough and smooth colony form. Bacteriophage susceptibility and resistance is associated with the presence or absence of filaments.

JULY 13, 1954

The dry matter content of starfish oocyte nucleoli. W. S. VINCENT AND A. H. HUXLEY.

The dry matter content of starfish oocyte nucleoli isolated in distilled water was determined by microinterferometry. The dry matter of nucleoli 4 μ in diameter averaged 85% (calculated as protein). With growth of the nucleolus the dry matter content decreased linearly with increasing nucleolar diameter, attaining a value of 40% in mature nucleoli 20 μ in diameter. Vacuoles in the nucleoli, occupying up to 50% of the total volume of the mature structure, contained less than 5% dry matter. Fragments of the same ovary from which the nucleoli were isolated were fixed and sectioned, and the concentration of protein in the intervacuolar areas of the nucleoli determined by microphotometry of the bromphenol blue and Millon stains. No change in concentration of protein in the intervacuolar regions of nucleoli 4 μ to 20 μ in diameter could be detected.

From these data we conclude that the starfish oocyte nucleolus is composed of highly concentrated, predominantly protein, materials in which are interspersed vacuoles containing less than 5% dry matter. Growth of the nucleolus appears to result primarily from accumulation of protein with a smaller contribution from the increase of vacuole volume.

Isolation of nuclei from Asterias eggs in the germinal vesicle stage and their DNA content. CELIA MARSHAK. No abstract submitted.

A theory of the biological role of DNA. ALFRED MARSHAK. No abstract submitted.

JULY 20, 1954

Influence of pH on the rate of respiration in Chlorella. E. P. NIELSEN. No abstract submitted.

"Univalent" antibodies. ALBERT TYLER.

Earlier experiments (1941 et seq.) showed that the fertilizin of sea urchin eggs could be converted by various methods into a non-agglutinating form termed "univalent" that retained its specific reactivity with sperm. Extension of the investigations (1945) to immune sera (rabbit anti-pneumococcus, anti-sheep cell, horse anti-diphtherial toxin, etc.) showed that photo-oxidation was an effective method of rendering the antibodies of these sera non-agglutinating or non-precipitating. In these experiments the ability of the treated antibodies to combine with antigen was tested primarily by the inhibition of a subsequent visible reaction to the addition of untreated antibodies. Another method of detecting non-agglutinating antibodies was later described by Coombs for the Rh system. This consists in adding an anti-human globulin serum to Rh-positive cells which have reacted with non-agglutinating human anti-Rh antibodies. Since the antibodies are globulins, then, having combined with the cells, the latter will be agglutinated by the anti-globulin serum.

If the antibodies that have been made univalent by photo-oxidation still retain their globulin specificity, the Coombs' test should be expected to be applicable to these, too. Experiments in collaboration with Dr. R. R. A. Coombs and M. Lorraine Fiset of Cambridge, England showed this to be the case. Rabbit anti-sheep cell sera were exposed to visible light in the presence of 0.2% eosin Y (water-soluble) until agglutinating action had been destroyed, the treatment being controlled also by measurement of O_2 uptake. Sheep cells upon treatment with such sera can be agglutinated by a goat anti-rabbit globulin serum. The univalent antibodies can also be conjugated with other substances, such as ovalbumin, and when now combined with the sheep cells, these can be agglutinated by an anti-ovalbumin serum. In addition, univalent antibodies against ovalbumin could be detected by the use of an antiglobulin serum. This procedure thus provides further opportunity for exploring the presence and role of univalent antibodies in allergy and pathogenic disease, and of investigating many other features of antigen-antibody reactions.

The activation of human plasminogen by streptokinase. WALTER TROLL AND SOL SHERRY.

Human plasma contains a proteolytic enzyme precursor termed plasminogen or profibrinolysin. The activation of this precursor to plasmin or fibrinolysin can be carried out by physical agents or specific bacterial kinases, *e.g.*, streptokinase. Two views have been proposed for the mechanism of the activation of plasminogen by SK. According to one view plasminogen is converted to a new substance, plasmin, by enzymatic activation. According to the other view the conversion of plasminogen to plasmin results from a stoichiometric interaction of SK with plasminogen. Our data reconcile the observations of these investigators. With the aid of synthetic substrates we have obtained evidence that plasminogen preparations contain two factors. One of these is activated by SK in a stoichiometric fashion to yield a plasminogen activator which enzymatically converts the second factor, plasminogen, to plasmin. The plasminogen activator has the properties of an enzyme splitting lysine esters, but is without action on arginine esters or casein. Plasmin, however, splits arginine and lysine esters and is proteolytic on casein.

JULY 27, 1954

Sympathectomy in frogs. JOSEPH PICK. No abstract submitted.

Sodium and the release of acetylcholine from sympathetic ganglia. O. F. HUTTER AND KRISTA KOSTIAL.

In suggesting the hypothesis that acetylcholine may be released from the nerve endings in exchange for sodium, Fatt and Katz called for verification by measurement of acetylcholine output. We have made an attempt in this direction by assaying the quantities of acetylcholine released from sympathetic ganglia perfused at various sodium concentrations.

Superior cervical ganglia of cats were perfused with eserinized Locke's solution, sodium being replaced by sucrose. So long as the perfusion fluid contained enough sodium for nerve impulse conduction no change in the output of acetylcholine could be detected. For example, stimulation of the preganglionic nerve for 5 minutes at 2 per second released as much acetylcholine after perfusion for ½ hour with a solution containing 30% of the normal sodium concentration, as when perfusing with Locke's solution. When the concentration of sodium was reduced to levels at which nerve conduction fails the output of acetylcholine decreased suddenly.

In some experiments ganglia were perfused with solution in which all sodium was replaced by sucrose, and the effect of depolarizing the nerve endings by increasing the potassium concentration was tested. The amounts of acetylcholine released by potassium under such conditions were comparable with the amount released by potassium in the presence of sodium.

These results indicate that in sympathetic ganglia sodium plays no part in the release of acetylcholine. There are, however, important differences between the anatomy of the nerve endings in ganglia and in muscle, and it is not impossible that our results are due to the presence of a diffusion barrier around the preganglionic nerve terminals which prevents the exchange of sodium for sucrose.

These experiments were made at University College London and at the Institute za Medicinska istrazivanja, Zagreb.

Electron microscopy of the sarcosomes in the heart. BRUNO KISCH.

The author emphasized first in 1951 the vital importance of the sarcosomes in the heart, being bearers of metabolic enzymes and responsible for the ability of the heart to work during a lifetime without longer rest. Biochemists all over the world have since proven the presence of these enzymes in the sarcosomes of the heart and the skeletal muscle.

Pictures of the inner structure prove a lamelliform arrangement of material. Sarcosomes of the mitochondrial type seem to prevail in the frog heart but not in the heart of mammals. The lamels are either crossing the sarcosomes in parallel layers or are arranged in concentric shell around one or different centers, or they are folded like a curtain. In some sarcosomes the content in cross-section looks like rings or loops or doughnuts.

A change of the appearance of the inner structure in connection with the functions of the sarcosomes is supposed, and attention was called to the startling similarity of the lamelliform contents of the sarcosomes and the arrangement of the myofilament in the muscle fibrils to Otto Lehmann's liquid crystals. After death the content of the sarcosomes disintegrates and more or less empty bags, the "ghost of sarcosomes," or their shadows, similar to the ghosts of red blood cells, remain, showing ridges or lamels inside a membrane not regularly arranged.

A not yet known new structure in the muscle fiber of the heart and the skeletal muscle was shown: a tube or fiber of ca. 300–500 Å width, straight or branching out between the myofibrils.

AUGUST 3, 1954

Identifying gene elements in Mormoniella. P. W. WHITING.

Of the many recessive eye-color mutant types found in this wasp, the majority occur at a single locus, R, consisting of two mutable elements, O and S. If either simple dominance or

blending occurs in the females compound for two mutant colors, the two mutations have affected the same element, either O or S. Codominance, complementarity, indicates that the two mutations have affected different elements, O and S, each change being recessive to its wild-type alternative in the other allele. A mutant male to be tested is crossed to a wild-phenotype female, compound for recessives in different elements, O.s/o.S. From the two different types of unfertilized eggs, O.s and o.S, laid by such a tester female there develop the two expected types of males. From the fertilized eggs there are produced females of two genotypes, both of which bear the new mutant paternal gene, m. If both female genotypes are wild phenotypes, O.s/O.S +/m and o.S/O.S +/m, the new mutation is not in R. If half the females are wild-type, the new mutation is in R, either in O, the females being O.s/m.S (wild phenotype) and o.S/m.S (recessive phenotype) or in S, the females being O.s/O.m (recessive phenotype) and o.S/O.m (wild phenotype). If none of the females are wild-type, the new mutation involves both elements, the females being O.s/m.m and o.S/m.m (both recessive phenotype). The fact that no R-locus mutants have been found siring only wild-type daughters suggests that there are not more than two mutable elements at this locus.

Some aspects of sexuality and genetics in desmids. RICHARD C. STARR. No abstract submitted.

On inherited and adapted rutin-resistance in Chlamydomonas. FRANZ MOEWUS.

Three strains of *Chlamydomonas eugametos* have been used in this investigation: *eugametos* (f. *typica*), characterized by a highly potent sexuality; *agametos*, characterized by its complete sterility; mutant *reru*, derived from *typica*. It could be shown that the sterility in *agametos* is due to a false biosynthesis of the flavonoid hormones, leading to the final product rutin instead of isorhamnetin or peonin. Rutin could be isolated from *agametos* cells. If rutin is offered to *eugametos* (10^{-10} g./ml.), it sterilizes completely this highly sexual strain. However, it has no sterilizing effect on the *reru*-mutant. In homogenates of *reru*-cells an enzyme system could be demonstrated which converts the offered rutin to the harmless ombuoside (= 7.4'-dimethylrutin). These results are only valid if the strains are grown under our usual standard methods. Their essential point is that the experimental material is obtained by spreading a suspension of *motile cells* on agar, where they undergo a log-phase of growth and develop within 2–3 weeks a monocellular film. Different results are obtained, when *eugametos* and *agametos* are grown by spreading thick layers of *non-motile cells* on agar, where they develop a slow growth. After one or more passages on agar (each time transferring the whole mass of non-motile cells onto the next plate), the physiological behaviour of *eugametos* and *agametos* is changed. They are characterized by heavy production of mucilage: they have become palmelloid. Palmelloid *agametos* shows now normal fertility, palmelloid *eugametos* is now rutin-resistant. In homogenates of both palmelloid strains the same enzyme system as in *reru*-mutant could be detected. In *agametos* the naturally produced rutin, in *eugametos* the offered rutin is converted to the harmless ombuoside. The appearance of this rutin-resistance is probably not due to spontaneous mutation. Experiments are in progress in order to explain this adapted character.

AUGUST 10, 1954

Ionic distribution and glycolytic factors in Fundulus. D. R. SHANKLIN.

Because of their relative abundance at Woods Hole, developing *Fundulus* embryos between Oppenheimer stages 22 and 27 were utilized in studies on cationic balance. During these stages there is a covering of ectoderm without orifices; circulation is complete and ectodermal-axial reflexes provide vital signs for biologic controls. During these stages the yolk occupies 50–60% of the total volume and is readily separable for distribution studies. Analyses indicate that magnesium is the principle intracellular ion with an inwardly directed gradient; sodium, potassium, and calcium having outwardly directed gradients. Yolk contains more free calcium than the embryo proper and less of the others; fluoride affects only the calcium content in yolk.

Bound fractions were determined. Influx of ions is linear, and at differing times for each ion, a plateau of exchangeability saturation is reached. From this plateau loss studies into sea water and osmotically balanced media lacking the ion in question were done. These losses are linear. Studies of this system with glycolytic inhibitors fluoride and iodoacetate were done, yielding changes in the slopes of uptake and loss, but not in the linearity. Since diffusion curves are hyperbolic, the linearity of these responses suggests a limiting transport system in both directions. Inhibitor studies show glycolytic tie-up with all four ion balances, the action towards sodium transport being in the direction of the gradient. A comparison of the potential maximum influx rate with the potential maximum outflux rate, measured against null gradient, in all cases yields a ratio statistically equal to the gradient, which would be expected if the cell were the cause of the gradient.

Some properties of mature and young erythrocytes. DAVID CHALFIN.

A comparison of red blood cell changes *in vitro* and red cell aging *in vivo* was undertaken. Erythrocytes from normal rabbits were incubated under bacteriological sterile conditions at 37° C. and followed for 30–40 hours for the *in vitro* studies. For the *in vivo* measurements, erythrocytes taken from a rabbit which had been rendered anemic by repeated hemorrhage were fractionated. The anemic blood was centrifuged and most of the reticulocytes were found to be concentrated at the top (centripetal portion) of the packed column of cells whereas the older cells were found at the bottom (centrifugal end) of the tube. Measurements were made on cation content, osmotic resistance, cell volume, hemoglobin content, density, dry weight and per cent water per cell for young and older red cells.

The younger red blood cells of rabbit are about one and half times larger, have a greater osmotic resistance, a lower density, a greater dry weight, and a higher K content than do older erythrocytes. The Na content remains relatively unchanged. The hemoglobin content remains constant as the cell matures but the cell volume decreases. The cation changes observed accompanying *in vitro* cell destruction: loss of K, gain of Na, loss of cation gradients, and increase in cell volume, are not the same as *in vivo* aging of the cell. Aging cells show a decrease in dry weight, a decrease in cation content as well as a decrease in cell volume, but a maintenance of the cation concentration and, most important, a maintenance of the cation gradients across the cell membrane.

Some new permeability constants for erythrocytes and their possible significance. M. H. JACOBS.

Under conditions and by procedures discussed in detail elsewhere it is possible to obtain for erythrocytes permeability constants (P) which measure the amounts of dissolved nonelectrolytes that cross unit area of the cell surface in unit time with unit difference of concentration between the inside and the outside of the cell. Approximately 100 such constants have been determined involving 16 species of mammals and 8 solutes. Erythrocytes of the groundhog and the muskrat are sharply separated from those of the other species and from most other known cells by P values, near 20° C., in cm./hour $\times 10^4$, of the order of several hundred, for all the solutes studied. Erythrocytes of the goat, sheep, beef, pig, horse, cat, dog, macaque, and chimpanzee showed values ranging from 0.4 to 3.9 for glycerol, 22 to 176 for ethylene glycol, and 6 to 51 for thiourea. These values are very similar to those reported by others for cells of about a dozen species of higher plants. Erythrocytes of man and the rat, rabbit, guinea pig and mouse showed values for glycerol from 12 to 120 for glycerol and 215 to 680 for ethylene glycol, but were very similar to those of the preceding group with respect to thiourea. The values for urea in the case of all species ranged from about 5,000 to 10,000, which may be compared with the average value of 3.7 for cells of 12 higher plants. For all the erythrocytes except those of the groundhog and muskrat the diethylene glycol and triethylene glycol values were fairly close to the averages for the species in question of 27 and 55, respectively. P values for diacetin were all of the order of several hundred and those for monoacetin, with a few exceptions, lay between those for diacetin and glycerol.

AUGUST 17, 1954

Activation of stretch receptors in Crustacea. CARLOS EYZAGUIRRE. No abstract submitted.

Effect of sympathetic stimulation on the slow skeletal muscle system of the frog. O. F. HUTTER AND W. R. LOEWENSTEIN.

In most skeletal twitch muscles of the frog, neuromuscular transmission proceeds, as a rule, with a considerable margin of safety: so long as the end-plate potential is large enough to set up a propagated action potential the muscle fibre gives a twitch. In such muscles sympathetic nerve activity can play a role only if a fringe of subliminally excited muscle fibres exists. The contractile mechanism of the multiply innervated slow skeletal muscle fibres of the frog is, however, activated in a different manner. Propagated action potentials are absent; instead there appears to be a close relation between the state of depolarization of the membrane and the contraction of the fibre. Since many similarities exist between the twitch fibre and the slow fibre system of the frog, it seemed likely that an agent facilitating neuromuscular transmission in twitch fibres will increase the tension response of unfatigued, untreated slow fibres. We have found that sympathetic stimulation increases the tension produced by the slow skeletal muscle fibres in iliofibularis on excitation of the small motor nerve fibres in the ventral roots.

Nature of neuromuscular facilitation by sympathetic stimulation in the frog. O. F. HUTTER AND W. R. LOEWENSTEIN.

It has long been known that in fatigued frog muscle stimulation of the sympathetic fibres causes a partial recovery of the twitch tension. This phenomenon has been ascribed to the diffusion of an adrenaline-like substance from the nerve endings of the vascular sympathetics to the muscle fibres. Since the twitch tension does not increase if the muscle is stimulated directly, it has been suggested that sympathetic stimulation causes a partial relief of neuromuscular block. Recording of muscle action potentials confirms this conclusion. In preparations partly blocked by fatigue or by too little initial stretch or by tubocurarine, sympathetic stimulation causes an increase in the voltage of the compound muscle action potentials, indicating the recruitment of fibres that have previously failed to respond.

In fully curarized preparations sympathetic stimulation, adrenaline and nor-adrenaline cause an increase in the amplitude of the end-plate potential. The increase rarely exceeds 10% but it can account for the observed restoration of transmission. The time course of the end-plate potential and the membrane potential of the muscle fibre are not detectably altered.

To localize the mechanism responsible for the neuromuscular facilitation the effect of nor-adrenaline on the depolarization of the motor end-plates produced by applied acetylcholine was studied. In a concentration of 2 in 10^8 nor-adrenaline increases the depolarization produced by a constant dose of acetylcholine by about 20%. It may be inferred from this that the increase in the nerve elicited end-plate potential is also due to a sensitization of the motor end-plates to acetylcholine.

In vivo measurements of the squid giant axon resting and action potentials. K. S. COLE AND J. W. MOORE. No abstract submitted.

GENERAL SCIENTIFIC MEETINGS

AUGUST 23–26, 1954

Abstracts in this section (including those of Lalor Fellowship Reports) are *arranged alphabetically by authors* under the headings "Papers Read," "Papers Read By Title" and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index (published in the December issue).

PAPERS READ

A new fibrous muscle protein. W. R. Amberson, J. I. White, H. B. Bensusan, S. Himmelfarb and B. Blankenhorn.

Extracts of rabbit skeletal muscle contain, in addition to actin and myosin, a third fibrous protein, provisionally called D-protein, which unites with myosin to form a complex, D-myosin. This complex can be detected on descending limbs of electrophoretic patterns given by mixtures of D-protein and myosin. It forms an elevation between faster moving free D-protein and slower moving free myosin. D-protein is obtained from ascending limbs by electrophoretic fractionation. Its solutions exhibit high viscosity and flow birefringence.

Solutions containing both D-protein and myosin have been studied in the Spinco analytical ultracentrifuge. The complex, D-myosin, is revealed by the use of the synthetic boundary cell introduced by Pickels, Harrington and Schachman. The lower two-thirds of the sectoral cavity is filled with a solution containing 0.4% D-protein and 0.4% myosin. The cup contains 0.4% D-protein alone. At about 8,000 r.p.m. this solution transfers from cup to sector, forming a layer which fills the upper third of the cavity. The run proceeds at 59,780 r.p.m. Two boundaries are initially seen, the upper due to D-protein alone, the lower to myosin alone. The lower boundary soon divides into two elevations. Free myosin sediments more rapidly. Myosin bound to D-protein sediments more slowly. After long running the upper D-protein boundary may reach and pass the more slowly sedimenting D-myosin elevation. A zone then exists which contains D-myosin alone, in the absence of both free myosin and free D-protein.

The strength of the union between D-protein and myosin is further demonstrated when the mixture in the lower part of the sector also contains 0.2% actin. The actin unites with all free myosin, forming actomyosin, which sediments rapidly. The elevation formed by D-myosin appears as before, sedimenting slowly. Actin is not able to cause the dissociation of D-myosin. Interactions involving all three fibrous muscle proteins may occur in the contraction-relaxation cycle.

Gel-formation in Limulus serum caused by a bacterial toxin, a protective mechanism of the host. F. B. Bang.

The intravascular clotting, and lethal effect, of a bacterial infection of Limulus was reproduced by a cell-free extract of boiled bacteria of this strain. The toxin caused a reduction in the number of cells in the peripheral blood of lobsters, spider crabs and in the coelomic fluid of Arenicola. Larger amounts killed lobsters and fiddler crabs.

Serum obtained from clotted blood in Petri dishes formed a gel within ten minutes after adding dilutions of "toxin," or living bacteria from the disease strain. Phase microscopy showed the bacteria to be immobilized by dilutions up to 1/8 of the serum. The gel was stable at room temperature for more than three weeks. Gels were formed by sera from which the hemocyanin was removed by centrifugation (35,000 r.p.m. for 6½ hrs.), but were not produced by heated sera (55° C.–10 min.). No young and not all old Limuli furnished sera which formed gels, but viscometry indicated quantitative rather than qualitative differences.

The bacterial toxin is non-dialyzable, and a similar effect was produced by a Shigella toxin of vertebrate origin.

Analysis of the luminescent responses in Mnemiopsis upon stimulation. Joseph J. Chang.

The luminescent cells in the eight meridional canals of the ctenophore, *Mnemiopsis leidyi*, respond only upon stimulation, either through nerves or directly. Using photomultiplier tube, amplifier, cathode-ray oscilloscope, and camera combination, the luminescent responses to various single and repeated electrical stimuli at room temperature as well as at other temperatures have been studied.

A large piece of luminous tissue responds by multiple flashes after a single stimulus when recorded locally. However, the response characteristics measured by light production from a small bit of tissue greatly resemble those of muscle twitches. A single square wave stimulus

elicits a single flash response. An increase in the stimulus voltage gradually increases the flash intensity. The same is observed when the duration of the stimulus is increased. At 23° C. a single flash curve has a latent period that varies considerably, a half-rise time of 35, a full-rise time of 60, a half-decay time of 48, and a 0.9-decay time of 114 milliseconds. Raising the temperature shortens the time course of a flash and lowering the temperature has an opposite effect. The decay of the flash intensity is logarithmic and the log decay constant vs. 1/T (Arrhenius plot) gives a straight line. Responses analogous to the summation of twitches, treppe, complete and incomplete tetanus of muscle are obtained by repetitive stimuli. Fatigue caused by multiple stimulation is very rapid in this luminous system. The luminescent response will traverse the length of a meridional canal with a conduction speed which averages 14 cm. per second at 23° C. Action potentials of fast frequency appear simultaneously with the luminescent response. Potential changes without a flash response have also been recorded along the canal.

Evidence for protein turnover in Amoeba proteus. ADOLPH I. COHEN AND SUK KI HONG.

Amoeba proteus in log phase growth were labelled by being fed S^{35}-labelled Chilomonas paramecium. The amoebae were then fed for 48 hours with unlabelled *Tetrahymena pyriformis*. After this period, sister cell pairs were isolated and one member mounted for radioassay. The corresponding sister cell was then allowed to feed on unlabelled Tetrahymena and after 4–5 cell generations, the total population resulting was mounted as a unit. After assaying, planchettes were extracted with cold 5% trichloracetic acid for one hour, dried, and re-assayed. The results showed a significant loss of total activity in both the non-extractable and extractable fractions during growth, while the percentage of activity that was non-extractable rose. Since cell death does not occur, turnover of some non-trichloracetic acid-extractable material or secretion of some sulphur-containing compound must account for these data. The chemical nature of the lost sulphur-containing compound is, as yet, unknown. Secretion of materials by amoebae is unknown. Preliminary experiments on starving amoebae show slight loss of total activity, if any, and less incorporation into the non-extractable fraction. Preliminary studies comparing starving nucleate and enucleate amoeba halves after six days, show a loss of activity for the nucleate halves. The enucleate amoebae show little loss, if any, and both halves show no significant incorporation into non-extractable material. Comparisons are complicated by the fact that heavily feeding and starving amoebae are far less motile than moderately fed or slightly starved amoebae. Enucleate and dividing amoebae are least active of all.

The contribution of the sperm aster to the first mitotic division. IVOR CORNMAN.

Mazia has demonstrated that the sperm aster, like cleavage asters, can be isolated by the technique that yields free spindles. From the similarity of material in these structures it is reasonable to conclude that the substance of the sperm aster contributes to the achromatic figure of the first cleavage. Evidence from colchicine-poisoned eggs supports this. If, by early treatment of *Arbacia* eggs, the sperm aster is prevented from forming, cleavage on removal to sea water is delayed much more than if treatment is begun after the streak has formed. In the latter case the streak material remains at the center of the egg and appears to be assimilated into the mitotic spindle on removal from colchicine. In *Asterias* eggs, where a threshold dose of podophyllotoxin has suppressed the sperm aster, the achromatic substance first materializes as many scattered spots around which there is subsequent multiple cleavage. A source of spindle material may well be the germinal vesicle, or even the nucleolus. There is enough protein in the nucleolus to form the achromatic figure and the nucleolus has solubility properties similar to those of the spindle (Vincent). Thus, the nuclear or nucleolar material is released when the germinal vesicle breaks down. The maturation divisions, unaided by the sperm aster, are able to muster only small spindles. The sperm aster, radiating throughout the egg, assembles the necessary nuclear and cytoplasmic components into the streak material at the center of the egg, where it is incorporated into the mitotic figure. The prominence of mitotic asters in large eggs undergoing holoblastic cleavage may also reflect this need for a system of collecting spindle material in an amount adequate to divide the entire protoplast.

Reconstitution of the intestinal tract in adult frogs, Rana pipiens. CHAUNCEY G. GOODCHILD.[1]

Experiments in which host organs were surgically altered to provide unusual implantation sites for parasites revealed that severed intestinal tracts of frogs possess ability not only to rejoin, but also to regain functional integrity. To study nutritional plasticity of trematodes, recta of frogs were disjoined from the ileum, washed free of debris, and used as implantation sites for gorgoderine trematodes. Operative procedure consisted of making a short incision on the right flank and lifting out the ileum and rectum. The ileum was tied two mm. anterior to the ileocolic valve and transected ahead of the stricture. The rectum was returned to the coelomic cavity and the ileum stitched to the flank to form an ileostomy. Although the ileostomy could be kept open by periodic attention, spontaneous healing by skin overgrowth usually occurred. When, in the latter, functional activity was indicated by appearance of normal fecal pellets, approximately two weeks after the operation, a series of operated frogs was studied. Even though the rectum and ileum had been purposely separated during the operation, after 5–6 days the short ileorectal stump was found adherent to the side of the ileum approximately 4 mm. from the body wall. During the next 7 days a gradual perforation of the ileum occurred at this point, and with concomitant closure of the ileostomy, the gut was reconstituted. The thread which had been used to tie the ileum was recovered in the rectal lumen. In other frogs simultaneous ileostomy and colostomy were performed in the same flank incision. In these animals, functional reunion between disjoined ileum and rectum was established within 12 days by a slight sinking in the openings and closure by a drawstring-like fold of skin growing inward from the margin. Specimens have been preserved for future histological examinations.

Survival of bladder flukes in new habitats. CHAUNCEY G. GOODCHILD.[1]

Anuran bladder flukes of the genera *Gorgoderina* and *Gorgodera* will survive when transplanted into urinary bladders of anuran species not naturally infected, but they quickly succumb when homoplastically or heteroplastically placed elsewhere in anuran hosts. Survival after implantation into urinary bladders of turtles and salamanders has now also been studied. Seven female painted turtles, *Chrysemys picta,* had 22 *Gorgoderina attenuata,* and 80 *Gorgodera amplicava* implanted into the true urinary and accessory urinary bladders. Flukes were not recovered from the latter after 24 hours. In the true urinary bladder some were recovered after 10 days; these were normal in behavior and were found only near the point of emergence of the bladder stalk. In the newt, *Triturus viridescens,* an intestinal immunity prevents bladder infection from ingested metacercariae, but implanted flukes were normal after an intravesical existence of two days. Fifty specimens of *Rana pipiens* were surgically altered to provide new implantation sites for *Gorgoderina attenuata,* taken from the same species of host. The majority of operations consisted of: 1) simple ileostomies, with the disjoined rectum forming an internal blind sac, 2) simultaneous ileostomies and colostomies, where the colon had anterior and posterior openings to facilitate cleansing and implantation, and 3) simple, midventrally-opening, small intestinal caeca separated from the rest of the gut. *Gorgoderina* placed in rectal caeca and in small intestinal caeca failed to live for 24 hours, but in recta open at each end some flukes lived in artificial frog urine for four days. Although bladder flukes failed to survive indefinitely, the new technique should be extremely valuable for elucidating host-parasite relationships of organisms regularly living in these gut regions.

Ca-initiated precipitation of nucleoprotein from homogenates of sea urchin eggs.[2] PAUL R. GROSS.

The role of calcium ions as initiators of cytoplasmic sol-gel transformations has been investigated at a biochemical level in homogenates of sea urchin eggs. The Ca^{++} is added to

[1] This investigation was supported by a grant from the McCandless fund of Emory University.
[2] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

homogenates made from eggs previously freed of their calcium. Water extracts of such homogenates show marked differences in protein and nucleic acid content dependent upon 1) presence or absence of calcium and 2) the amount of Ca added and the time of incubation after calcification. In these experiments, the fraction of the homogenate containing the large pigment granules was removed by centrifugation at 0° C. for 10 minutes at 1000 × g. Experiments were performed upon the supernatants which contain yolk as well as all of the smaller particles.

The precipitation of protein is a first-order reaction. With Ca added to a final concentration of 0.010 *M*, a homogenate may lose 10% of its water-soluble protein within 15 minutes and 18% within 60 minutes. PNA, as determined by the orcinol reaction, precipitates more rapidly, reaching a level of 26% precipitated within 60 minutes. It is assumed as a working hypothesis that the precipitated material is a nucleoprotein, richer in PNA than is the homogenate as a whole. The traces of DNA present in the homogenate cannot contribute significantly to these effects.

Electrophoretic experiments indicate that the material precipitated comes mainly from the largest fraction of the water-soluble proteins, and that this material has an unusually high mobility (-13×10^{-6} at pH 7.6 and ionic strength = 0.1). The high density of negative charges also suggests that the precipitating material is a nucleoprotein.

Electron micrographs of whole homogenates and of the two fractions show that the aggregating particle is 400 A.U. in diameter. These particles can dissociate into spheres of high electron density 60 A.U. in diameter.

The calcium, as measured by the method of Fales (1953), is not precipitated to a measurable extent with the macromolecular materials.

Some observations on "plasma clots" and "fibrin clots." [1] PAUL R. GROSS, ALVIN M. KAYE, DELBERT E. PHILPOTT AND PETER RIESER.

In the terminology adopted by Lorand, blood clots appear in one of two forms; one, produced by the action of thrombin on fibrinogen in the absence of calcium and/or plasma factors, is urea-soluble. This is the "fibrin clot." The "plasma clot" forms when calcium and a plasma factor are present, and is urea-insoluble.

The plasma clot is generally more opaque than a fibrin clot. This fact has led us to seek differences in clot structure at the submicroscopic level.

For these experiments, undiluted citrated sheep plasma, as well as plasma diluted 1:6 with 0.9 per cent NaCl, was clotted with either thrombin (10 units/ml.) or $CaCl_2$ solution (2 mg. Ca/ml.). For the electron microscopic observations, collodion-filmed nickel grids were dipped for 15 seconds in the clotting mixture and fixed in 0.2 per cent phosphotungstic acid at different times relative to the time of clotting in the original vessel. Afterwards, the material was washed in distilled water.

On only one occasion were large fibrin fibers showing the characteristic 230 A.U. periodicity obtained. This system was one clotted with thrombin and was more dilute than the systems described above.

Ordinarily, no periodicity was shown in the clots. Both the fibrin clots and the plasma clots were tight networks of fibrils of smaller diameter than those observed in clots formed from purified fibrinogen.

The fibrin clots were usually fine networks, and showed the substrate uniformly coated with material. The plasma clots were always more strongly cross-linked, showed marked syneresis on the substrate, and had areas of nodular, highly electron-dense material which were never found in the Ca-free systems.

X-irradiated plasma (100,000 r) produced fibrin clots whose rate of dissolution in 5 *M* urea was markedly accelerated.

A further study of isometric mechanical responses of the anterior byssus retractor muscle of Mytilus edulis to electrical stimuli and mechanical stretch. WILLIAM H. JOHNSON. See abstract under "Lalor Fellowship Reports."

[1] This investigation was supported by a research grant from the U. S. Atomic Energy Commission, administered by L. V. Heilbrunn.

The nature of the oxygenation reaction in hemocyanin. THEMIS ASKOUNIS KLOTZ AND IRVING M. KLOTZ.

The consensus (Redfield, 1933, 1950) has been that copper exists in the cuprous state in hemocyanin, both in the deoxygenated and oxygenated forms. It has been pointed out (Klotz, 1950, 1952), however, that the spectrum of oxyhemocyanin shows marked similarities to those of certain cupric proteins. Attempts to resolve this conflict by magnetic and polarographic methods have not given unequivocal answers.

The status of the copper has now been established as follows. A dialyzed solution of hemocyanin, and one of biquinoline in glacial acetic acid, are deoxygenated by bubbling with purified nitrogen. The two solutions are then mixed under nitrogen. For oxyhemocyanin the same solutions are used, but they are kept exposed to air. When cuprous ion is present, a red copper-biquinoline complex is formed immediately; cupric ion gives no color. A typical set of results with plasma from *Busycon canaliculatum* is:

	Non-oxygenated	*Oxygenated*
Cuprous copper	$6.5 \times 10^{-4} M$	$2.9 \times 10^{-4} M$
Total copper	$7.9 \times 10^{-4} M$	$7.2 \times 10^{-4} M$

It is apparent first that copper is almost entirely in the cuprous state in the non-oxygenated protein. (The difference between total and cuprous copper may be contributed by biologically inactive protein.) Of greatest interest, however, is the observation that only approximately one-half of the cuprous copper is transformed to cupric during oxygenation. Since one molecule of oxygen is held by two copper atoms, it is clear that the active site consists of copper in each of two valence states. This information together with spectroscopic comparisons enables one to draw a structure for the active site which explains much of the behavior of hemocyanin.

Thus the oxygenation reaction in hemocyanin is markedly different in molecular nature from that in hemoglobin.

Controlled observation of hatching in Fundulus heteroclitus. ROGER MILKMAN.

In fish (except the grunion) hatching is normally far too unpredictable to observe satisfactorily. Controlled observation of Fundulus eggs has been made possible by specifically preventing hatching until desired. Normal development is otherwise unaffected.

Eggs are placed in about 100 cc. of sea water in a flask within a few days of fertilization, and oxygen is bubbled in. The flask is then stoppered until several days after the expected hatching date. Upon washing in sea water the eggs hatch in a few minutes (24° C.).

Although extra oxygen itself has an inhibitory effect on hatching, shaking oxygenated water removes the effect; but shaking does not remove the inhibition caused by the water the fish have been in, nor does temporarily making the pH 3 or 12, or heating to 65° C. Boiling removes the inhibition.

The hatching sequence begins with the simultaneous initiation of mouth activity, excretion of granular material from the anus, and contraction of the yolk sac to about ⅓ its former volume. Once this has occurred, hatching is assured, even though the eggs be replaced in inhibitor water. About 7½ minutes and 10 intrachorional somersaults later, the embryo breaks out.

During these 7½ minutes, the chorion changes from a smooth, tough, relatively thick coat, which keeps its shape, to a rough, weak, thin flaccid covering. The chorion originally has two layers, the outer being left as the thin, weak layer. The inner layer is quickly destroyed by an enzyme, but the fish must break out of the outer one. If a fish is anesthetized just before it is due to hatch, the thin layer remains intact indefinitely, although the enzyme retains its activity for days *in vitro*.

Because of its autonomy, its integration of rapid motor processes, and its result, the hatching process may be thought of as behavior.

The effect of oxygen on the frequency of x-ray induced mutations in Habrobracon sperm. W. E. MURPHY.[1]

In the cross used in this experiment about 66% of the eggs are fertilized and diploid (female-producing); 34% are not fertilized and haploid (male-producing). When untreated females are mated to males x-rayed with sub-lethal doses, the number of sons remains unchanged while daughters are decreased due to dominant lethals induced in sperm. Each surviving daughter, bred unmated, demonstrates in her eggs (all unfertilized) her genotype in respect to recessive embryo lethals. If she carries none, all her eggs hatch, if one, 50% hatch, if two (non-linked), 25% etc. When heterozygous for a post-embryo lethal or a visible mutation, half her sons show it.

Two groups of males were x-rayed (4234 r), one in air, the other in nitrogen during irradiation, 100 seconds. Males exposed to nitrogen alone for twenty minutes bred as did controls, 96% hatchability of F_1 eggs, no mutations. Irradiated males were mated immediately to untreated females and then removed so that only sperm mature at time of exposure were tested. Hatchability of F_1 eggs was 38.4% for the air group and 45.5% for the nitrogen ($7.1 \pm \sigma_{diff.}$ 1.0), dominant lethal rate was 92.8% for former, 79.7% for latter ($13.1 \pm \sigma_{diff.}$ 0.03). Number of females produced per day per female was 5.6 ± 1.7 in controls, 0.9 ± 1.0 in nitrogen and 0.34 $\pm$ .6 in air. Recessive embryo lethals per F_1 female were 1.2 when sperm had been x-rayed in air and 0.55 when in nitrogen; recessive post-embryo lethals were carried by 7.7% of former, 8.6% of latter; visible mutations by 23.0% of former and 6.25% of latter. Tests of F_1 females are still being made. These differences when significant are smaller than those observed by Anna R. Whiting (unpublished) for eggs of Habrobracon irradiated under comparable conditions.

Adenosinetriphosphatase activity of spermatozoa. LEONARD NELSON.[2]

$MgCl_2$ (10^{-4} *M*) increases the rate of ATP hydrolysis by whole Mytilus sperm which has been washed in filtered sea water (F.S.W.) and suspended in isotonic KCl to 220% of the control. All concentrations of $CaCl_2$ decrease the activity. If the sperm is washed twice in F.S.W., and then four times in isotonic KCl, Ca^{++} (10^{-3} *M*) increases the ATP-ase activity to 130% while Mg^{++} (5×10^{-4} *M*) raises the activity to 240% of the control. In combination the two cations antagonize each other.

The flagellar components of homogenized sperm have at least 13.5 times greater ATP-ase activity than the head fraction on a nitrogen content basis. Optimum ATP-ase activity in veronal buffer lies between pH 8 and 8.5. The temperature optimum occurs at approximately 30° C., and the activation energy of the crude tail suspension, over a range of 21°–27° C., is 13,700 Cal./degree/mole. When ATP concentration is limiting, only the terminal phosphate is liberated. The enzyme activity is proportional to the quantity of tail suspension present in the reaction mixture.

In some respects, this material resembles muscle ATP-ase. The tail fragments may be precipitated out of sea water or isotonic KCl by the addition of 5 to 20 volumes of ice-cold glass-distilled water. Amino acid composition of tail and muscle protein hydrolysates will be compared as well as electrophoretic and ultracentrifugation measurements on the purified preparations.

Where is the plasma membrane of the unfertilized egg of Arbacia punctulata? A. K. PARPART AND P. C. LARIS.

The literature contains frequent reference to a plasma membrane immediately inside the vitelline membrane of the unfertilized egg of *Arbacia punctulata*. In addition, the cortical granules first described by Harvey, 1911, have been described as embedded in a gel-cortex just

[1] This work was done while working with Dr. Anna R. Whiting under a grant from the Phi Beta Psi Sorority.

[2] Partially supported by a grant from the Faculty Research Council of the University of Nebraska.

inside the plasma membrane. There is also evidence that while this egg cell is permeable to ethylene glycol, it is only very slightly permeable to glycerol and relatively impermeable to erythritol, xylose, glucose and sucrose.

We have observed the cortical granules of the unfertilized Arbacia egg by means of the television microscope under a variety of experimental conditions. These granules swell and "explode" within a few seconds after these cells are exposed to isosmotic solutions of ethylene glycol, glycerol and erythritol and in 50% of eggs in xylose and glucose. Following the explosion of these granules a membrane is lifted from the egg in a manner analogous to the formation of the fertilization membrane including the marked churning of the cytoplasm immediately adjacent to the cortex. The remainder of the egg, however, does not increase in volume except in the case of ethylene glycol. Also these cortical granules cease to refract light when the egg is exposed to isosmotic sucrose though this can be reversed by replacing the sucrose with sea water. Such reversal can be repeated many times. The refractive index of this sucrose solution must be similar to that of the granules. The sucrose must penetrate the gel-cortex, but not the cortical granules.

It is therefore concluded that the plasma membrane of the mature unfertilized *Arbacia* egg must be at the inner margin of the gel-cortex. The cortical granules are outside the plasma membrane and they must lie in the gel-cortex between the vitelline membrane and the plasma membrane.

Electron microscopic investigation of bumble bee wing muscle. DELBERT E. PHILPOTT.

This investigation was carried out using material sectioned with a microtome designed by the author. The microtome consists of a motor-driven specimen holder which rotates in a vertical circle. The knife advance and holder are complete in one unit, thus simplifying the construction and operation. The coarse and fine mechanical advance is produced from one gear by dropping a worm gear (100:1 ratio) down on the coarse gear advance. This moves a rod whose opposite end holds the glass knife. The rod is electrically heated to produce the final advance of 2–300 Å per section. It has proved an economical and simple microtome to operate and construct. The bee wing muscle was fixed in neutral buffered osmic acid entering from the anterior end and fixing *in vitro*, thus preventing isotonic contraction. The tissue was then removed, dehydrated in isopropyl alcohol and embedded in methyl and butyl methacrylate (1:3) for sectioning. No cross band structure (A or I bands) has been seen either in longitudinal or cross sections. The protofibrils have been found to be packed in very regular hexagonal array. The diameter of one protofibril is 220 Å ± 15 Å and the mean distance from center to center of protofibrils is 350 Å, but disarrangement in fixation can vary this considerably. The sarcomer length is 2 μ and width of Z line is 800–1000 Å. An M line and thin H line also appear on all sections. The protofibril appears to get slightly narrower in front of the Z membrane and larger in it. The protofibrils are densely packed in an osmophilic substance in the Z membrane. The per cent shrinkage is estimated from 10 to 30%. This would increase the distance between protofibrils in the living state even beyond 130 Å. The Z band is tightly packed so probably there is but little shrinkage in it.

Cation antagonisms. D. R. SHANKLIN AND PHILIP B. ARMSTRONG.

The classical work of Loeb on salt antagonisms was done on *Fundulus* embryos. Believing that newer methods would facilitate studies of this kind, *Fundulus heteroclitus* embryos were subjected to varying concentrations and osmotic strengths of the chlorides of calcium, magnesium, potassium, and sodium. Exchanges of these cations across the ectoderm of Oppenheimer stages 22 to 27 were studied by radiotracers Na^{22}, Na^{24}, K^{42}, and Ca^{45}; magnesium was studied by a modified TCA titan yellow method for total Mg, corroborated by a method using 4-p-nitrophenylazoresorcinol, and exchangeable magnesium by a modified molybdivanadate method for phosphate; flame spectrophotometry was used for total sodium and potassium, and calcium was measured by a chelate titration of murexide complex with ethylenediaminetetracetate. That the site of salt-acid antagonism is the ectoderm and not the chorion was shown by Armstrong (1928) and by Armstrong and Shanklin (1952); thus salt antagonisms were studied at

the ectoderm. These studies show that the salt effects are not all antagonistic. In several instances, *e.g.*, Na for K, and Mg for Ca, facilitation is profound. These results are tempered by knowledge of active transport mechanisms in *Fundulus* for influx and outflux (Shanklin, 1954). In osmotically balanced media, magnesium uptake is greater in all cases than is predictable from summation of unit action of the other ions on magnesium, while for Ca, K, and Na, the uptake is less than predictable. These results are in line with active mechanisms yielding a gradient inward for Mg, and outward for the others. The orders of action do not follow Hofmeister's lyotropic series, but appear to be specific properties of ions. The activity of ions in this system is Ca > K > Mg > Na, which is inverse to their concentration, and in the order of their atomic mass.

Cell movement and the axial gradient in Tubularia. MALCOLM S. STEINBERG.

The axial gradient in Tubularia is expressed by a decreasing rate of hydranth regeneration as one proceeds proximad down the stem. During regeneration there is a period of tissue movement toward the regeneration site, followed by the differentiation of a hydranth there. These experiments test the hypothesis that all differences in regenerative rate lie in the movement phase. In the first experiment 5-mm. sections were cut from stems, the distal cut being made 2 mm. below the base of the hydranth. Only sections of the widest and narrowest diameter were used, 20 wide pieces constituting one group and 20 narrow ones constituting the other. Time for regeneration was 48.5 hours for the narrow group and 59.1 hours for the wide group. Time for tissue movement was 22.5 hours for the narrow group and 32.7 hours for the wide group. Time for differentiation was 26.0 hours for the narrow and 26.4 hours for the wide group. In the second experiment twenty 10-mm. sections were cut similarly and ligated in the middle. Time for regeneration was 33.3 hours distally and 46.5 hours proximally. Time for movement was 21.4 hours distally and 33.0 hours proximally. Time for differentiation was 11.9 hours distally and 13.5 hours proximally. Repetition of this experiment on another colony gave 41.3 hours for regeneration distally and 75.2 hours proximally. Time for movement was 25.8 hours distally and 59.9 hours proximally. Time for differentiation was 15.4 hours distally and 15.3 hours proximally. The results show that while stems from different colonies differentiate at different rates, variations in regenerative rate between individual stems within the colony or between different regions of the same stem are due to differences in the rate of tissue movement and not in the rate of differentiation. Other experiments show that the movement is due to amoeboid ectoderm cells, which indicates that the axial gradient in Tubularia is in reality a reflection of the activity or orientation of these cells.

Sedimentation constants, viscosity, and diffusion of the fertilizins of Arbacia and Echinarachnius.[1] ALBERT TYLER, ALAN BURBANK AND JAMES S. TYLER.

Solutions of the fertilizins of *A. punctulata* and *E. parma* were prepared by the previously described (Tyler, 1948, 1949) methods. Sedimentation velocities were determined in the Spinco analytical ultracentrifuge, using relatively dilute (0.1% and 0.2%) solutions in pH 9.02 borate buffer ($\mu = 0.1$) forArbacia and pH 7.02 phosphate buffer ($\mu = 0.1$) for Echinarachnius. These gave average s_{20}'s of 7.09×10^{-13} cm./sec. unit field for Arbacia and of 4.67×10^{-13} for Echinarachnius, as compared with a previously (1949) obtained value of 6.3×10^{-13} for *Strongylocentrotus purpuratus.*

The intrinsic viscosities are 0.942 for Arbacia and 0.895 for Echinarachnius. Calculated as spheres, the molecular weights would be 82,800 for Arbacia and 54,000 for Echinarachnius.

Attempts were made to determine the diffusion coefficients by use of spreading of refractive index boundaries both in electrophoretic cells and in the ultracentrifuge (at low speeds). Since solutions of high concentrations gelate, the measurements were attempted with the relatively small areas and peaks obtained with dilute solutions, and these gave variations of over threefold. Taking as a first approximation 2.1×10^{-7} cm.^{2}/sec. for Arbacia gives a molecular weight of 300,000, and an axial ratio (as unhydrated prolate ellipsoid) of 28 : 1.

[1] Supported in part by a grant (C-2302) from the National Cancer Institute, Public Health Service.

The electrophoretic mobilities of the fertilizins of Arbacia and Echinarachnius.[1] ALBERT TYLER, ALAN BURBANK AND JAMES S. TYLER.

Electrophoretic mobilities of the fertilizins of *A. punctulata* and *E. parma* were determined in the Tiselius apparatus in dilute (0.2%) solutions. The values obtained (in $cm.^2/sec./volt \times 10^4$) are, for Arbacia, 1.99 in pH 7.02 phosphate buffer ($\mu = 0.1$), 1.57 in pH 4.84 acetate buffer ($\mu = 0.1$) and 2.02 in pH 2.0 HCl–KCl buffer ($\mu = 0.05$), and, for Echinarachnius, 1.82 in pH 7.02 phosphate buffer ($\mu = 0.1$). The value for Arbacia at pH 2 seems anomalous. The other values are of the same order as those previously (1949) obtained for Strongylocentrotus and show the fertilizins to be highly acidic glycoproteins of low isoelectric point.

Studies on urocanylcholine. V. P. WHITTAKER AND I. A. MICHAELSON.

Urocanylcholine, first discovered by Erspamer and coworkers in certain Mediterranean molluscs, notably *Murex brandaris*, has now been identified spectroscopically in two related North Atlantic species, *Urosalpinx cinerea* and *Thais lapillus*. The active material was extracted from the whole organisms by the usual trichloracetic acid procedure. This extract contained much material with a non-specific U.V. absorption. Accordingly an amount of extract equivalent to about one gm. of tissue was poured onto a weak acid ion exchange resin previously buffered to pH 4.3 and eluted with 0.1 *M* NaH_2PO_4 followed by 0.1 N HCl. The U.V. absorption and biological activity on the frog rectus preparation of successive fractions was determined. During the elution with phosphate, a biologically inactive component with intense U.V. absorption at 270 mμ made its appearance in the early fractions; this was tentatively identified as homarine on the basis of (a) the wave-length of the absorption peak, (b) the invariance of the peak with pH. The next material to appear was acetylcholine, identified by differential assay on the frog rectus and clam heart and by its similar behavior on the column to synthetic acetylcholine. On elution with acid the pH of the effluent fell, and a component with strong U.V. absorption and biological activity made its appearance. The spectrum of this compound was identical in the range 240–350 mμ with Erspamer's curve for urocanylcholine; on acid or alkaline hydrolysis, activity was lost and the spectrum was replaced by that of urocanic acid. Urocanylcholine accounted for 80–90% of the acetylcholine-like activity of the original extracts and its concentration in *Thais* and *Urosalpinx* was 360 and 230 μgm./gm. fresh tissue, respectively.

PAPERS READ BY TITLE

Fluctuation in the form of the daily rhythm of O_2-consumption in fiddler crabs.[2] M. F. BENNETT, F. A. BROWN, JR. AND H. M. WEBB.

The O_2-consumption of two species of fiddler crabs, *Uca pugnax* and *Uca pugilator*, was measured continuously for about 45 days from June 23 to August 5 under constant conditions of temperature and illumination. The temperature was 19.5° C. and the illumination was less than 1 ft. c. When the form of the persistent daily cycle of O_2-consumption was determined for each of several semilunar periods it was found that the form of these exhibited a cycle of change through the month. During the two semilunar periods commencing with the first quarter of the moon, the rate was generally high through most of the day with maxima about 3 A.M., 9 A.M., and 3 P.M., and with a predominant minimum about 8 to 10 P.M. For the period commencing at new moon, the three maxima were more clearly evident. For the periods beginning at the first quarter and full moon, the 3 P.M. maximum became progressively more dominant with a lower rate of O_2-consumption during the morning hours. In every period the lowest rates of the day occurred during the evening hours. These observations strongly suggest that the crabs possess a cycle of O_2-utilization about a synodic month in length.

[1] Supported in part by a grant (C-2302) from the National Cancer Institute, Public Health Service.

[2] This work was supported in part by contracts NONR-09703 and NONR 122803 with the Office of Naval Research.

Some physiological properties of frog sperm.[1] G. S. BERNSTEIN.

Sperm of *Rana pipiens* and *R. clamitans* were obtained from extirpated testes mashed in buffered (3×10^{-4} *M* phosphate, pH = 7.00), 8.7% (v/v) Ringer's solution. Large pieces of testicular tissue were removed by filtration through cheese cloth. The motility of washed and unwashed sperm from both species was reversibly inhibited 10 to 20 minutes after being placed in an oxygen-free (alkaline pyrogallol) chamber. HCN (10^{-3} and 10^{-2} *M*) reversibly inhibited motility within 6 minutes. Added glucose did not prevent the inhibition of motility by lack of oxygen or HCN. The sperm were treated with inhibitors in further attempts to characterize their metabolism. Fluoride, iodoacetate, azide, and mixtures of these substances (all at 10^{-3} *M*) did not affect motility in three hours. The failure of these substances to inhibit motility may be due to their high degree of dissociation, and hence their low penetration into the sperm, at pH 7.00.

There are several available substances which may be used as substrates by frog sperm. *R. clamitans* sperm contain 1.2×10^{-7} γ of lipid phosphorus per cell. Unwashed suspensions of *R. clamitans* sperm contain 10 to 25 γ of reducing sugar per ml. Paper-chromatographic analyses showed that only one reducing sugar is present; this is an aldohexose (reddish-brown color with *p*-anisidine HCl) having an R_f value identical with that of glucose.

These data indicate that frog sperm obtain energy for motility from aerobic metabolism, possibly involving the use of phospholipid or glucose.

The response of fibrillar flight muscle to rapid release and stretch. E. G. BOETTIGER AND E. FURSHPAN.

Wing movements in some insects are produced by antagonistic fibrillar muscles, both stimulated in flight at frequencies that produce tetanus in the isolated muscles. We previously suggested that the speed of shortening releases the tension which does not reappear during the subsequent stretch. To test this theory, a preparation of the longitudinal flight muscle of the bumble bee was used.

This muscle tetanizes at 40 stimuli per second; produces up to 40 grams tension; shows very strong summation; contraction takes about 250 msec. and relaxation twice as long. A muscle 5–6 mm. long can shorten 0.3 mm. from *in situ* length. Stretching above this length 0.1–0.2 mm. increases active tension. Probable shortening during normal flight is 0.1 mm. A fast release of 0.1 mm. produces almost complete loss of tension. The tension returns to that characteristic of the new length in the usual manner.

When a passively stretched unstimulated muscle is stretched following a release, the tension rises in phase with the stretch. A stimulated muscle can be stretched after release with the return of only 40–50 per cent of the original tension. The remaining tension returns following the attainment of full length. This property does not depend on the time after release as much as on the stretch itself.

Persistent rhythms of O_2-consumption in carrots and potatoes.[2] F. A. BROWN, JR., R. O. FREELAND AND M. F. BENNETT.

Sections of carrots and pieces of potatoes bearing eyes, each weighing about five grams, were investigated continuously for a synodic month and hourly values of O_2-consumption were obtained. Under the conditions of constant temperature and constant very low illumination there were continuous variations in the rate of O_2-uptake but there was no overt similarity of the patterns from day to day. When, however, the data for the whole lunar period were examined for the existence of a rhythmic component of primary solar frequency, an unequivocal daily

[1] Aided by grants from the Lalor Foundation, the Research Committee of the Faculty of the University of Delaware, and by a contract (Nonr 160–015) between the University (Dr. R. R. Ronkin) and the Department of the Navy, Office of Naval Research.

[2] This work was supported in part by contracts NONR-09703 and NONR 122803 with the Office of Naval Research.

cycle was apparent for each plant. The carrots exhibited a principal maximum about 5 A.M. and a principal minimum about noon; the potatoes showed two conspicuous maxima, one at 5 A.M., and the other at 3 P.M., with a principal minimum about midnight. An analysis of the data to demonstrate any persisting primary lunar rhythm showed the potatoes to have a minimum at lunar zenith, followed by a principal maximum eight hours later and a lesser minimum at lunar nadir followed by a lesser maximum about four hours later. For the carrots the lunar cycle was tetramodal with the principal maxima following lunar zenith and nadir by nine and five hours, respectively. The presence of a lunar cycle of O_2-consumption was also apparent in the demonstration that for both plants the diurnal cycle calculated for the semilunar period, May 12–26 gave the major maximum in the A.M., and for the period May 27–June 9, in the P.M.

The primary lunar rhythm of O_2-consumption in the fiddler crab.[1] F. A. Brown, Jr., M. I. Sandeen and C. L. Ralph

The O_2-consumption of two species of fiddler crabs was determined continuously for more than forty days and the data analyzed to learn the form and phase relationships of any persistent primary lunar or tidal rhythm which might exist. The data, for analysis, were subdivided into five semilunar periods, each beginning with the day of one of the quarters of the moon. In both species, for the first semilunar period, there were found simple sinusoidal rhythms with maxima of O_2-consumption at the times of low tide in the habitat from which the crabs had been collected. In the period covering the second and third weeks, the amplitudes of the lunar cycles were greatly reduced with maxima not only at the times of low tide but also with new maxima appearing about halfway between. For the remaining three periods freshly collected *Uca pugnax* were added from time to time; the lunar cycles tended to remain more or less bimodal. For the *Uca pugilator* the third fifteen-day period of analysis, utilizing some of the initial lot of animals and some which had been freshly collected, had bimodal lunar cycles. During the succeeding fifteen-day period continuing with the same collection of crabs the cycles continued to be bimodal, but by the last semilunar period had become again unimodal but now with minimum rate of O_2-consumption occurring about the time of lunar zenith and nadir. It appeared, therefore, that the crabs kept in the laboratory gradually shifted the phases of their tidal rhythm from a minimum about the time of high tide, to the time of lunar zenith and nadir which in this instance was six hours out of phase with the habitat high tide.

Solar and lunar rhythms of O_2-consumption in the seaweed, Fucus.[1] F. A. Brown, Jr., M. I. Sandeen and H. M. Webb.

Tips of thalli of the brown alga, Fucus, aggregating about five grams, were placed in each of seven transparent respirometer vessels suspended in a black-lined constant-temperature bath provided with a constant illumination of 1 ft. c. at the water surface, and the O_2-consumption measured continuously over a period of more than 15 days. Fresh plant material was substituted every four to six days. The rate of O_2-consumption fluctuated greatly during the period of the study. The over-all average was about 49 ml./Kg./hr. but the rate during the period varied as widely as from about 30 to about 100 ml./Kg./hr. When the data were analyzed for the presence of a rhythmic component of primary solar frequency between the dates July 29, the day of a new moon, and August 12, about the time of a full moon, there was found to be a daily cycle which was essentially bimodal. There were two nearly equal maxima, one about midnight and the other about noon. Minima were seen at 6 A.M. and about 3 P.M. From a minimum to a maximum involved about a 10% increase in rate. When the primary solar rhythm was rendered random and the data analyzed for any rhythmic component of primary lunar or tidal frequency, there was found present a low amplitude primary lunar rhythm during this same period. The lunar rhythm was one in which there were broad maxima separated by minima occurring at the times of lunar zenith and nadir. From maxima to minima in the lunar cycle involved an increase of only about 6% in rate of O_2-utilization.

[1] This work was supported in part by contracts NONR-09703 and NONR 122803 with the Office of Naval Research.

Osmotic resistance of dogfish and tautog erythrocytes. DAVID CHALFIN.

Osmotic resistance measurements were made of the erythrocytes of dogfish and tautog at two pH's, 5.8 and 7.5. The isotonic solution for dogfish is 0.35 *M* NaCl and for the tautog is 0.21 *M* NaCl. The fragility curves for the dogfish cells are different from those of the tautog and in each case the curves at pH 5.8 are different from those at 7.5. In tautog, the cells appear less osmotically resistant at pH 5.8 than at pH 7.5. The tautog hemoglobin is precipitated at pH 5.8 but not at pH 7.5 so that the amount of measured hemoglobin at the acid pH never approaches the amount in solution at the alkaline pH. In addition, however, the amount of hemoglobin in the hemolysates of presumably completely hemolyzed cells varied slightly with salt concentration at pH 5.8 but not at 7.5.

In dogfish the cells appear slightly more osmotically resistant at pH 5.8 than at pH 7.5. This hemoglobin, in contrast with that of the tautog, is not precipitated at the acid pH. The shape of the osmotic resistance curve at pH 7.5 has a bimodal character; *i.e.*, the dogfish cells are grouped into two main classes of either size, membrane resistance or some other governing factor. At pH 5.8 the amount of hemoglobin in the hemolysate goes through a maximum as the tonicity is increased from distilled water towards isotonicity. At this maximum (ca. 30% of isotonicity) the amount of hemoglobin measured is almost equal to that found in basic solutions of similar tonicities. This resulted in what appeared to be less hemolysis in distilled H_2O than in 5% of isotonicity, less at 5% than at 10% of isotonicity and so on up to the maximum at 30% of isotonicity (0.105 *M* NaCl). Further study indicated that this was due to the fact that the hemoglobin which had come out of the cells adhered to the ghosts at pH 5.8 and the amount of this adhesion was dependent on the ionic strength of the hemolysate.

Mitotic inhibition by caffeine and x-rays. RALPH HOLT CHENEY AND ROBERTS RUGH.

Comparative effects of different caffeine molarities upon the development of the egg of *Arbacia punctulata* were stated in 1948 by Cheney. In 1940, Henshaw reported effects of x-ray irradiation on Arbacia as being most severe in the prophase stage. The current experiments submitted unfertilized eggs in filtered sea-water (SW) to x-rays at the rate of 6,000 roentgen units per minute for intervals totalling 1,000 r to 300,000 r; and/or pretreatment of 30 minutes in various caffeine molarities, prior to insemination with non-treated sperm in SW. The fertilization process and developmental rate were observed continuously for two hours and at intervals to pluteus larva. Study was made of the effect of a given amount of irradiation and of a given caffeine molarity, separately and in combination. The chronology and extent of delay were recorded in the different mitotic phases of the first cleavage. Any over-all time delays in subsequent development were noted with reference to the organism's attainment of pluteus formation.

In agreement with Henshaw, the greatest effect (slowing up) by x-ray irradiation was observed during prophase. Our observations demonstrate clearly that the chemical agent, caffeine, is also most effectively inhibitory during amphiaster transitions of mitotic prophase. Caffeine and x-rays appear to affect mitosis at the same stage, *i.e.*, prophase. Therefore, we considered the possibility of synergistic, summation, or even antagonistic action of these two agents. Since no such effects were observed when these agents were applied in combination, it is presumed that the mechanism of their effect is different. Since it is known that x-rays alter the physical make-up of chromosomes and that caffeine affects the cortical granules and surface forces of the egg, we may suggest that x-rays delay cleavage by their effect on chromosomes and the first effect of the caffeine is primarily on the cytoplasm.

Uptake of certain hexoses by Arbacia larvae. G. H. A. CLOWES, A. K. KELTCH AND C. P. WALTERS.

To supplement previous studies by the authors upon hexokinase activity of unfertilized and fertilized Arbacia eggs, hexose uptake of Arbacia larvae has been measured. Fertilized eggs were allowed to develop in sea water at 25° C. for 24 hours with continuous gentle stirring, yielding a homogeneous population of plutei; these were collected by gentle centrifuging, resuspended in sea water containing the desired hexose at .0004 *M*, and incubated for two hours

at 20° C. in Warburg vessels with shaking at 70 cycles per minute. Glucose was taken up at an average rate of approximately 250 micrograms per hour per gram wet weight of larvae; this rate was not affected by dinitrocresol at concentrations from 4×10^{-6} *M* to 1.0×10^{-5} *M*. The use of mannose and galactose was small as compared to glucose; fructose did not appear to be used at all; exact values for hexoses other than glucose cannot be stated because the Nelson method is not completely satisfactory for these sugars. When the hexose added was fructose, there was a net increase in reducing substances during incubation. The oxygen consumption of the larvae, as in the case of unfertilized and recently fertilized eggs, rose to a maximum at 8×10^{-6} to 1.6×10^{-5} *M* dinitrocresol and decreased at higher concentrations; the addition of any hexose to the medium did not alter the basal oxygen consumption or the form and height of the curve with dinitrocresol.

Retention of phosphate label by starving Amoeba proteus. ADOLPH I. COHEN.

Amoeba proteus were indirectly labelled with P^{32} via feeding them *Tetrahymena pyriformis* which had been grown in media containing inorganic P^{32}. Single amoebae may be labelled to the extent of 500–1000 counts per minute after 48 hours of growth on such labelled ciliates. After growth on unlabelled ciliates for 48 hours to obviate label loss by defecation, single amoebae in a standard fluid volume and in a standard container were assayed daily, while living, for radioactivity. Such measurements were made over 10 days of starvation in various media or during feeding. Feeding amoebae divide and the total resulting population is then counted simultaneously as a unit. Starving amoebae usually show one division (presumably from reserves) and occasionally a second division, even after visible size loss and loss of reduced weight. In confirmation of the work of Frederich-Freksa, no loss of activity was found for the fed, growing population. Surprisingly, no detectable loss of activity was likewise found for the starving group. The amoeba medium employed has 8 mg. KCl, 4 mg. $MgSO_4 \cdot 7H_2O$, and 12 mg. $CaHPO_4 \cdot 2H_2O$ (saturated) per two liters of Pyrex-distilled water. The omission of phosphate ion, the addition of glucose (.004 *M*), the addition of sodium glycerophosphate (.004 *M*), and various combinations of these did not alter the findings. The method appears capable of detecting losses of over 15%. The loss of reduced weight during the starving period is of the order of 50%. Since James has reported loss of ribonucleic acid during starvation of whole amoebae, an attempt was made to fractionate amoebae and look for shifts in label distribution. The attempt to get a reliable fractionation procedure has thus far been unsuccessful and efforts along this line are continuing.

The effect of tetrodotoxin and related substances on cell division.[1] PIERRE COUILLARD.

It was suggested by Yudkin in 1945 that tetrodotoxin, the poison from the ripe ovary of tropical tetrodont fishes, can also occur in *Spheroides maculatus*, the common "puffer" of the North-Atlantic coast.

Ripe ovaries of *S. maculatus* contain a dialyzable factor which is active in preventing germinal vesicle breakdown in the oocyte of Asterias and cleavage in the eggs of Asterias, Chaetopterus and Arbacia. Like tetrodotoxin (which is also dialyzable), this factor is lacking in immature or spent ovaries. Crystalline tetrodotoxin (kindly supplied by the Sankyo Co., Tokyo) was shown likewise to inhibit both germinal vesicle breakdown and cleavage.

Spent ovaries do contain an antimitotic substance, but it is not readily dialyzable. Furthermore, crude extracts of such ovaries will markedly enhance germinal vesicle breakdown in starfish oocytes. In all cases the effects are not due to changes of pH.

Previous work, notably experiments with inhibitors such as periodate, points to this non-dialyzable antimitotic agent as being a muccopolysaccharide, having a heparin-like effect on blood clotting, possibly homologous with a number of such substances found by Heilbrunn and his co-workers in the ovaries of many vertebrate and invertebrate forms including 12 local

[1] Holder of a Special Scholarship, National Research Council of Canada. Part of this investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

species of fishes. Protein-free polysaccharides, containing from 5 to 8% glucosamine, have indeed been extracted in crude form from puffer ovary. Though they are metachromatic and occasionally exhibit anticoagulant action, they have failed, so far, to show antimitotic activity. This could be due to the lack of an essential protein cofactor (as recently shown by Rathgreb and Sylvén in the case of Shear's polysaccharide).

A conjugating strain of Tetrahymena vorax.[1] ALFRED M. ELLIOTT AND ROBERT E. HAYES.

Two clones of *Tetrahymena vorax* were isolated in April, 1954, from a small roadside stream near the village of La Chorrera in the Republic of Panama. Species identification was made from Feulgen and silver nitrate preparations following the taxonomic system proposed by Corliss. The two clones are considered identical and are here designated as strain RP. They are maintained in axenic culture.

All animals possess a typical macronucleus and usually one micronucleus, or rarely two. No amicronucleate individuals have been observed. It appears to have a higher number of chromosomes, of approximately the same size, than the conjugating strains of *T. pyriformis*, WH6 and WH14.

Strain RP exhibits the polymorphic life cycle characteristic of the species. Transformation from a pyriform microstome stage to the cannibalistic macrostome form occurs readily in starved cell suspensions.

Animals washed in distilled water and allowed to starve for several hours conjugate (self) within the clone. The stages of conjugation are essentially like those of *T. pyriformis*. At the end of the process conjugates separate and give rise to viable exconjugant clones, which are sexually immature.

Tetrahymena from Mexico, Panama, and Colombia.[1] ALFRED M. ELLIOTT AND ROBERT E. HAYES.

One hundred forty-two fresh-water samples from Mexico (37), Panama and the Canal Zone (81), and Colombia (24) were examined for *Tetrahymena*. The habitats included rivers, ponds, lakes and irrigation ditches. They ranged in elevation from sea level to 10,000 feet, and in temperature from approximately 8° to 35° C. Of these 142 samples 30 were positive, and from these 277 clones of *Tetrahymena* were isolated and established in axenic cultures. Two proved to be *T. vorax;* all others, *T. pyriformis.*

None of the 277 clones were able to grow when any one of the 19 nutrilites essential for strain E were absent from the medium. Their growth characteristics in the standard peptone-tryptone stock medium resemble those of strain E.

The nuclear picture varied among the different clones. For example, from two habitats in Mexico and 10 in Panama, all contained micronuclei, whereas 5 other Mexican and 5 Panamanian habitats yielded only 11 out of 41 clones that contained micronuclei.

All clones were tested for their mating reaction. Conjugating strains were found in 12 of the 30 habitats studied, all of which belong to variety 2 of Gruchy's mating system for *Tetrahymena pyriformis* (except the *T. vorax* strains, which are selfers). Conjugating strains were also found from 5 additional habitats but they have not been identified as to variety. All mating strains possess micronuclei. No amicronucleate clones have been observed to conjugate.

X-irradiation of sexual strains of Tetrahymena.[1] ALFRED M. ELLIOTT, ROBERT E. HAYES AND J. ROGERS BYRD.

The non-conjugating, amicronucleate strain E (*T. pyriformis*) is highly resistant to x-rays. The mating types, WH6 and WH14, on the other hand, do possess micronuclei and should furnish more favorable material for detecting radiation damage to the genetic system.

[1] This investigation was supported in part by a research grant (PHS G3588) from the National Institutes of Health, Public Health Service.

Distilled-water suspensions of vegetative cells of both mating types were exposed to graded doses of x-rays employing the Wichterman-Figge plastic radiation chamber. At 50,000 r intervals samples were removed and placed in peptone-tryptone stock culture media. Following 72 hours of incubation the amount of growth was determined. No animals survived 650,000 r and the LD 50 was computed to be approximately 400,000 r. Surviving cells which received 200,000 to 600,000 r, inclusive, were permitted to conjugate and a search was made for evidences of cytological abnormalities. The conspicuous anomalies were failures of the homologous chromosomes to pair properly.

In order to determine the relative sensitivity to x-rays at various stages in the life cycle, mating types were first permitted to conjugate and reach the crescent stage of prophase of the first prezygotic division, and then exposed to graded doses of x-rays. Samples were withdrawn at 5000 r intervals. Twenty pairs were isolated from each sample and placed in stock media in depression slides. After 24 hours of incubation they were transferred to tubes. Ninety-six hours later relative growth was determined. No cells survived 400,000 r and the LD 50 was computed to be approximately 300,000 r. Cells that survived conjugation gave rise to viable cultures which appeared to be normal as regards their cultural reaction in stock medium.

Excitation in nerve cells of the lobster stretch receptors. C. EYZAGUIRRE AND S. W. KUFFLER.

The stretch receptors in the lobster (*Homarus americanus*), recently described by Alexandrowicz, offer a suitable preparation for the study of many aspects of nervous activity. Each stretch receptor unit consists of a fine muscle bundle which receives one sensory neuron and one or more motor and inhibitory nerves. The sensory discharge of stretch receptors is controlled by passive stretch, by motor nerve impulses activating the muscle elements of the receptor and by nerve inhibitory action. The cell body of the sensory neuron, situated in the periphery, sends dendrites into the muscle elements. Contraction or stretch of the receptor muscle causes excitation of the dendrites. By recording with intracellular electrodes from the nerve cell many events leading up to excitation were analyzed. By stretch deformation the dendrites become depolarized and thus they serve as generators for the subsequent events in the nerve cell. The generator potential within the dendrites is transmitted electrotonically to the adjoining cell body which fires propagated nerve impulses at a critical level of its membrane potential. The dendrite potentials are graded dependent on the extent of stretch and accordingly they "drive" the discharge in the adjacent cell body at different rates. Some receptor neurons adapt quickly and thus serve to register transient tension changes, while others adapt slowly and discharge for the entire duration of stretch. It is noteworthy, however, that in both neurons the level of the membrane potential in the cell body of the sensory neuron reflects faithfully the changes in the "generator" located in the nearby dendrites. Thus the level of the cell membrane potential can be "set" for long periods and thereby the excitability of the cell is adjusted. The dendrite-nerve cell system resembles an electrical transducer and by recording the membrane potentials, minute contractile changes in the receptor have been detected.

Inhibitory activity in single cell synapses. C. EYZAGUIRRE AND S. W. KUFFLER.

The sensory neuron of the lobster stretch receptor makes contact with the contractile receptor elements through dendritic processes. These dendrites also carry synapses from "accessory" fibers (Alexandrowicz). These fibers have now been identified as inhibitory and some of the mechanisms of inhibition have been studied. The action of inhibitory synapses directly affects the "generator" potential in the dendrites and thus indirectly controls the excitability of the adjacent cell body and the discharges of the sensory neuron. Inhibitory nerve impulses cause potential changes which depend on the level of the membrane potentials (M.P.). If the M.P. level is reduced by a constant stretch, inhibitory nerve impulses will repolarize it; in the absence of stretch with the M.P. near its "resting" state, inhibition may cause no detectable hyperpolarization, but may even set up a small depolarization. The main inhibitory action, therefore, appears to consist in driving the membrane potential of a cell

towards its "equilibrium" level. The effect of each inhibitory impulse, reflected in the membrane change, rises to its peak in a few msec. and decays in about 30 msec. Inhibitory activity can stop the sensory discharges in spite of strong stretch on the receptor, by first repolarizing the cell membrane and then "holding" or "stabilizing" it, thus preventing depolarization and excitation. The antidromic spread of a nerve impulse into the nerve cell may also be prevented by inhibition, but it is thought that this occurs only when the safety margin of propagation near the axon-soma boundary is low. These results are closely related to those of Katz and Fatt on crustacean nerve-muscle junctions and to those of Eccles and collaborators on the cat's spinal motor neuron.

Parasites in starved guts in well-nourished hosts. CHAUNCEY G. GOODCHILD.[1]

Relative dependence of gut parasites on food absorbed from the lumen as contrasted to food absorbed from the mucosa are unanswered questions of fundamental importance in parasitology. The rationale and operative procedure of the preliminary technique herein reported had been perfected before an identical procedure used in hormone studies, the so-called Thiry-Vella fistula, was discovered. Fourteen white rats, under ether anesthesia and after 24 hours starvation, had 2-cm. incisions made through the integument and body wall of the lower right flank. The small intestine was pulled through the opening and an 8–15 cm. section, with mesentery intact, was cut free. The intestine was then rejoined and the ends of the isolated section sewed at the ends of the incision. After 48 hours the animals were again given food and water. The U-shaped fistula was flushed periodically with mammalian Ringer's until healing had occurred in approximately 5–7 days. One or two tapeworms, *Hymenolepis diminuta*, from donor white rats, were then implanted, in mammalian Ringer's, deep into the fistula by means of a flexible polyethylene tube of proper bore. The parasites established themselves and survived in some hosts without exogenous food for 3 days. This technique, for the first time, permits placing gut parasites in starved guts, but in well-nourished hosts. It should also permit sterilizing the fistula with suitable antibiotics and administration of selected metabolites to determine relative dependence of many gut helminths, without microbial contaminants, on absorption from lumen as contrasted to absorption directly from the mucosa of the host.

The effect of insulin on the respiration of the sponge, Microciona prolifera. E. E. GORDON AND MELVIN SPIEGEL.

It was shown by Krebs and Eggleston (1938), using a pigeon liver suspension, that insulin could effect an increase in O_2 uptake. We have been able to demonstrate this action of insulin on an invertebrate—the sponge, *Microciona prolifera*.

O_2 consumption was measured by the conventional Warburg technique. Apical portions of sponge were cut into 2–5 mm. pieces, allowed to dry for approximately two minutes on absorbent paper and weighed. Either K pyruvate (0.001 *M*), glucose (0.0056 *M*), or Na_3 citrate (0.001 *M*) in sea water was used as substrate. Experiments were also performed without added substrate. Four units of regular insulin (Iletin, Lilly) containing 0.2% phenol as preservative were added to each of the appropriate vessels. As controls, distilled water or 0.2% phenol was added in place of insulin. The vessels were incubated for two or three hours at 37.2° C. in an air atmosphere. At the end of this period, aliquots of the medium were removed for chemical determination.

In the presence of insulin, O_2 uptake is increased an average of 16%. Controls utilized an average of 0.116 μl. O_2/mg. wet wt./hr. With added insulin the average O_2 uptake is increased to 0.134 μl./mg. wet wt./hr. Neither phenol, in concentrations present in the commercial insulin, nor any of the added substrates had an effect on O_2 utilization. No significant utilization of any added substrate by control and insulin-incubated preparations was detected. Determination of keto acids in the pyruvate-incubated preparations revealed a production of these acids.

[1] This investigation was supported by a grant from the McCandless fund of Emory University.

The carbohydrate metabolism of the slime mold, Dictyostelium discoideum, during development.[1] JAMES H. GREGG AND RUTH D. BRONSWEIG.

The slime mold, *Dictyostelium discoideum*, derives its energy solely from an endogenous food supply during morphogenesis. During the transformation from the migrating pseudoplasmodia to the mature sorocarps certain nitrogenous components are utilized. It was of interest to investigate the role of the carbohydrates during morphogenesis, not only from the standpoint of determining the amount of energy-yielding substrate utilized but also to determine the source of the extracellular cellulose which is deposited around the stalk and spore cells of the mature sorocarp.

The dry weights of the slime mold amoebae, migrating pseudo-plasmodia, mature sorocarps, spores and stalks were determined by weighing on a quartz helical balance. The tissues were then analyzed for total reducing substances which were assumed to be primarily reducing carbohydrates. The total reducing substances/dry wt. show a consistent increase from the vegetative amoebae to the mature sorocarps. The stalk component of the mature sorocarps showed a greater increase in total reducing substances than the spores. The increases in total reducing substances/dry wt. may reflect a possible loss in dry weight which might occur during the development of the slime mold. However, it seems clear from the data that protein is the ultimate major substrate utilized for purposes of obtaining energy and satisfying other morphogenetic requests rather than the carbohydrates.

Electrical and mechanical manifestations of muscle activity caused by rapid cooling. RITA GUTTMAN AND MILTON M. GROSS.

The anterior byssus retractor muscle of *Mytilus edulis*, previously potentiated with potassium (from 39 to 91 millimols KCl added to sea water), was subjected to rapid cooling. The potential difference between one end of the muscle immersed in 0.26 *M* KCl and the other experimental end of the muscle was measured by means of a galvanometer used as a null point instrument. Virtually isometric contractions were recorded on a kymograph. Depolarization occurs on rapid cooling, the degree of depolarization depending upon the amount of cooling. Contractions occur whenever the cooling is rapid and the amount of depolarization exceeds a critical threshhold value. If the cooling is gradual, the amount of depolarization is about the same, but no contraction occurs. After the action potential has been initiated by rapid cooling (from about 22° C. to 2° C.), recovery occurs in about 80 minutes, if the cold temperature is maintained. This recovery consists of a return to a steady state with a P.D. about 50% of the original value.

Changes in the protoplasm during maturation.[2] L. V. HEILBRUNN AND WALTER L. WILSON.

It has long been known that the protoplasm of eggs in the germinal vesicle stage is more viscous than it is after the germinal vesicle breaks down, and it has generally been assumed that the liquefaction of the protoplasm following germinal vesicle breakdown is due to the release of substances from this structure. In Chaetopterus eggs, germinal vesicle breakdown is induced by the entrance of the eggs into sea water. Goldstein in 1953 showed that as the eggs left the ovary and entered sea water, the protoplasm of the cortex became less rigid. Calcium is apparently released, and this calcium activates a proteolytic enzyme system. Such a protease might clot the protoplasm and it might also liquefy the protoplasm by dissolving some viscous constitutent of it. Actually, in the Chaetopterus egg it does both. The entrance of the egg into sea water is immediately followed by a sharp increase in protoplasmic viscosity. At 21°, the time relations are as follows. Within 30 seconds after the eggs enter sea water the viscosity doubles, but this increase is very transitory, for one minute after the eggs have entered

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the sea water the viscosity is approximately the same as in the ovarian egg. From then on the viscosity drops rapidly so that 5 minutes after the entrance of the eggs into sea water the viscosity is only ¼ as great as in the ovarian egg. The germinal vesicle does not break down until 7 minutes after the eggs have entered the sea water. Thus, apparently, a calcium-activated protease can cause both gelation and liquefaction of protoplasm, and the liquefaction does not depend on the breakdown of the germinal vesicle.

Some properties of the regenerating limb blastema cells of salamanders.[1] H. HOLTZER, G. AVERY AND S. HOLTZER.

Recent analysis (S. Holtzer, 1954) of the morphogenetic mechanisms in tail regeneration in salamanders suggested that: (1) the cells of the regeneration blastema form cartilage primarily, the muscle originating directly from the muscle of the stump, and (2) the regenerating spinal cord by patterning the mesodermal tissue is responsible for the morphology of the tail. The following experiments were designed to ascertain whether (a) cells of the salamander limb blastema likewise differentiated primarily into cartilage and (b) the mesodermal tissues of the limb were responsive to the patterning effects of the spinal cord.

Clusters of 3 or 4, 9-day limb blastemata were introduced into tunnels in the dorsal fin of a salamander host and allowed to remain for two weeks. The blastemata fused, grew and eventually formed large masses of organized cartilage. Long bones, metacarpals, phalanges and joints could be observed. No differentiated muscle fibers were present. Thus limb blastema cells grown under quasi-tissue culture conditions differentiated primarily into cartilage.

The following preparation served to test the reactivity of the limb mesoderm to the action of the spinal cord. The humerus was removed and a piece of spinal cord of similar length substituted. Following a two-week healing period, the limb was amputated through the graft. Though many bizzare structures regenerated, for the most part they were clearly limb-like. In addition to generalized enlargement of the regenerated tissue, often projections consisting of epidermis and a cell-sparse connective tissue formed. These were strikingly similar to fin tissue and probably attributable to the activity of residual neural crest derivatives included with the spinal cord. Failure to observe tail structures (*e.g.*, segmented muscles, vertebral cartilages, etc.) in these experiments suggests the spinal cord is incapable of: (1) modeling limb mesoderm in a fashion analogous to its action on trunk mesoderm, and (2) inducing vertebral cartilages from limb mesoderm.

The inductive role of the spinal cord in tail regeneration in the salamander.[1] SYBIL HOLTZER.

Though parallels between embryogenesis and regeneration have been pointed out, the phenomenon of induction has never been included in such a list. Analysis of tail regeneration in the salamander indicates that induction is truly a component of that process and can be shown to operate in a variety of experimental situations. In this particular system the spinal cord serves as inductor while the somitic mesoderm responds. The interactions between the two are as follows: In normal tail regeneration the notochord does not regenerate; rather, directly below the motor surface of the spinal cord, appear segmented cartilages, the centra, which later develop neural and haemal arches. That such cartilages are not derived from notochordal cells is shown by the fact that they may be obtained by (a) amputation of cord-muscle deplants into the dorsal fin, (b) grafting of nerve cord directly under the skin of flank and tail, (c) displacement of nerve cord to a more dorsal position in the midline. No centra are produced by amputated deplants of notochord or notochord and muscle in the dorsal fin.

It is to be emphasized that the centra form only along the motor surface of the spinal cord, regardless of the D-V orientation of the latter with respect to the muscle. Presumably the cartilage promoting activity resides exclusively in the motor half of the spinal cord.

Other results indicate that the muscle in the regenerated tail buds off from that in the stump, unilateral deficiencies there being preserved in the regenerate. Muscle will regenerate a

[1] Supported by a U.S.P.H. and a Woods Hole-O.N.R. grant.

limited amount in the absence of nerve cord but will form no cartilage. Contrary to previous reports tail and limb regeneration systems are comparable in that both can regenerate skeletal elements though such elements are absent in the stump.

Ecology of the intertidal fauna of the Woods Hole region. ROBERT STEARNS HOWARD.

At Gansett Beach in Woods Hole, Massachusetts, the amphipod *Orchestia* is the most abundant macroscopic organism at the low-tide mark. Its dark-brown color provides camouflage in the wet sand. Up-beach, the whitish *Talorchestia* replaces *Orchestia*, its color likewise offering camouflage in the dry sand. Along rocky or sandy shores, the green crab *Carcinides* is the dominant organism, being replaced in muddy areas by the mud crab *Neopanope*. Halacarid mites, nereid annelids, the gastropod *Littorina*, the barnacle *Balanus*, the pelecypods *Mytilus* (in rocky areas) and *Brachidontes* (in muddier areas) are abundant at the low-tide mark. These apparently require submersion most of the time. Up-beach (or higher on rocks in the same area), the barnacle *Balanus* (with porous plates) is replaced by *Chthamalus* (with non-porous plates). Between the high- and low-tide lines is a narrow band (often less than three feet wide) where pseudo-scorpions (*Pselaphochernes?*) occur. Here the collembolan *Anurida* (found also nearer to or walking on the water) is found in abundance under rocks. Trombidiid mites, pseudoscorpions, and the pselaphid beetle *Brachygluta luniger* feed on the collembolans here. The collembolans feed on detritus. Farther back (ca. 18 feet from the low-tide mark), only gammarid crustaceans are found. Four feet back (ca. 22 feet from the water), the seaside earwig *Anisolabis maritima* is found along with the gammarids. Four feet farther back (ca. 26 feet from the water), a few typically inland terrestrial forms appear, such as millipedes, centipedes, etc. The earwig *Anisolabis* is abundant from this region to the drift line, but is not found farther back.

Comparative ecology and behavior of two sympatric earwigs, Forficula auricularia Linnaeus and Anisolabis maritima (Gene). ROBERT STEARNS HOWARD.

Though sympatric, the European earwig *Forficula auricularia* Linnaeus and the seaside earwig *Anisolabis maritima* (Gene) are separated ecologically. *Anisolabis* is restricted to the intertidal zone; *Forficula* is never found there, though a distance of only ten feet may separate the two. *Anisolabis* lives among the pebbles and debris near the high-tide mark. *Forficula* is most abundant where thick sod abuts a solid surface. *Anisolabis* is highly cannibalistic even when food is abundant. *Forficula* may eat live insects (and even fight over them), but it displays no cannibalism. In *Forficula*, there are two morphological types of males, the "regular" type and the *forcipata* type with greatly enlarged forceps (over twice the size of the "regular" forceps); 317 (45%) of 702 males examined were of the *forcipata* type. Both types were found in all aggregations examined. Trios (one female, one "regular" male and one *forcipata* male) tested for dominance and subordination proved the *forcipata* dominant in 36 (64%) of 56 cases where dominance was demonstrated; 46 additional cases were inconclusive. In both species, the forceps are used in fighting, threatening, defense, courting and copulation. *Forficula* uses the forceps also to help fold the flying wings under the tegmina. *Anisolabis* is wingless. In *Anisolabis* the female always dominated the (somewhat smaller) male. In *Forficula*, the sexes are about equal in size, and neither sex dominated. The only regular domination displayed was of the *forcipata* type over the regular type of male.

A case of apparent physiological competition between ethylene glycol and glycerol. M. H. JACOBS.

Evidence has been given elsewhere that the entrance of glycerol into the erythrocytes of man and several other mammals takes place by two processes: (1) a relatively slow one of simple diffusion through widespread non-specialized pathways, as in other cells, and (2) a rapid one, apparently limited to small specialized portions of the surfaces of the erythrocytes in question. The rapid process can be reversibly inhibited by moderate acidity, traces of

copper, and other agents that have little effect on the slower one. The 2-carbon homologue of glycerol, ethylene glycol, enters all cells so far studied with great ease, presumably by simple diffusion. Studies of permeability constants, however, have shown that in erythrocytes possessing the rapid mechanism for glycerol the rate of entrance of ethylene glycol also tends to be more rapid than in the case of other cells, and further that this "extra" rapidity, like that of glycerol, is sensitive to acidity and traces of copper. The following observations seem to suggest that ethylene glycol can successfully compete with glycerol for the "rapid" pathway. (1) In the presence of ethylene glycol the entrance of glycerol into human erythrocytes may be greatly slowed. (2) The same and even higher concentrations of ethylene glycol have little effect on the entrance of substances believed to follow unspecialized pathways (thiourea, diethylene glycol, triethylene glycol) or specialized pathways different from those used by glycerol (anions). (3) Ethylene glycol does not slow the entrance of glycerol into beef erythrocytes, which lack the fast process. (4) Ethylene glycol has little or no retarding effect on the entrance of glycerol by the slow process into human erythrocytes in which the fast one has been inhibited by acidity or copper.

Valence changes in the oxygenation of hemerythrin. I. M. KLOTZ, R. A. RESNIK AND T. A. KLOTZ.

By analogy with hemoglobin it is generally assumed that the iron in hemerythrin is in the ferrous state, both in the deoxygenated and oxygenated forms. This view has been strengthened by the observation (Marrian, 1927; Florkin, 1933; Roche, 1933) that oxidizing agents, which presumably transform the iron into the ferric state, convert hemerythrin into methemerythrin which no longer combines with oxygen. Since oxidizing agents give misleading results, however, as regards the state of copper in hemocyanin, some doubt is raised as to the significance of similar experiments with hemerythrin. Furthermore, the effects of oxidizing agents give no insight into the status of the metal in oxyhemerythrin.

The status of the iron has now been established as follows. A solution of crystalline hemerythrin in sea water and one of *ortho*-phenanthroline in glacial acetic acid are deoxygenated by bubbling with purified nitrogen. The two solutions are then mixed under nitrogen. For oxyhemerythrin the same solutions are used, but they are kept exposed to air. When ferrous ion is present, a pink-colored, iron-phenanthroline complex forms slowly; ferric ion produces no color at all.

With oxyhemerythrin, all of the iron is released in the ferric state. With deoxygenated hemerythrin almost all of the iron is in the ferrous state; approximately 25% may be in the ferric form. It is possible, however, that this small fraction of ferric ion is contributed by biologically inactive protein, for hemerythrin which has been allowed to stand at room temperature in air for several days, until it loses its capacity to combine with oxygen, shows only ferric ion even in the absence of oxygen.

It becomes apparent, therefore, that unlike hemoglobin, hemerythrin actually undergoes a change in the valence state of its iron in the oxygenation reaction.

Activity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in relation to glycolytic enzymes of Arbacia eggs. M. E. KRAHL, A. K. KELTCH, C. P. WALTERS AND G. H. A. CLOWES.

Glucose-6-phosphate (GP) and 6-phosphogluconate (PG) dehydrogenases of Arbacia eggs were determined from rates of triphosphopyridine nucleotide (TPN) reduction at 23° C. in the Beckman spectrophotometer at 340 mμ. Egg fractions were obtained as previously described. Final concentrations in the test system were: KCl, 0.08 *M*; glycylglycine, 0.025 *M*; $MgCl_2$, 0.03 *M*; TPN, 0.00005 *M*; egg protein, 0.1–1 mg.; substrate, as desired; total volume, 2–3 ml. Rates are average values for the first two minutes after substrate addition and refer to the extract from one gram eggs. At 0.001 *M* GP, the rate of TPN reduction by the homogenate of unfertilized or 30-minute fertilized eggs was 2.5–3.5 micromoles per minute, enough to support a rate of oxygen consumption 25 times that observed for unfertilized, and 6 times that for fertilized, eggs; 95 per cent of this activity was recovered in the supernatant fraction after

centrifuging at 20,000 g for 30 minutes. With the supernatant fraction the concentrations of GP and PG for half maximal activity were each approximately 0.00004 *M* for the respective enzymes. Maximal activity with PG as substrate was 50–60 per cent of that with GP. During oxidation of GP by the supernatant fraction of unfertilized eggs the pentose formed was at a rate 0.3–0.5 that of TPN reduction. Rates of TPN reduction with other substrates at 0.001 *M* were: glucose-1-phosphate, 0.4; fructose-6-phosphate, 2.6; fructose-1,6-diphosphate, 0.8, indicating presence of phosphoglucomutase, isomerase, and a fructose-1,6-diphosphatase, respectively. Reduction of diphosphopyridine nucleotide in presence of fructose-1,6-diphosphate and adenosine diphosphate was 0.1–0.2 micromoles per minute, indicating that the glycolytic pathway over aldolase and oxidizing enzyme can metabolize GP at about 5 per cent the rate at which it can be oxidized by the TPN system derived from unfertilized or fertilized Arbacia eggs.

The effect of cortisone and of corticotropin (ACTH) on the rate of cell division.[1] VALY MENKIN AND MAX PEPPER.

Earlier studies by one of us (V. M.) have indicated that previous to fertilization, exposure of *Arbacia punctulata* ova to cortisone and ACTH reduces the incidence of cell division. Cortisone acetate administered 30 to 40 minutes after fertilization delays the rate of cleavage. The delay seems proportional to the actual concentration of the corticosteroid used. The amounts employed varied from 0.5 mg. to 2.5 mg. per 10 cc. of sea water. There follows within the first hour and a half a delay in the rate of cleavage ranging from 20% to 41% in the appearance of the 3–4 (or over) blastomeric stage. When the corticosteroid was added 1–10 minutes following fertilization, the effect was even more marked, the delay being 70% in the series studied. The use of a soluble salt of sodium cortisone phosphate (courtesy of Dr. W. Umbreit, Merck Co.), several minutes after fertilization, in concentrations of 7–10 mg. likewise delayed the cleavage in 6 experiments to an average of 39%. On the following day the controls usually displayed free swimming plutei larvae, whereas the experimental ova, as a rule, manifested a definite retardation in the development of their plutei. Testosterone propionate (2.5–5 mg.) as a control steroid failed to induce any appreciable delay in cleavage, the average retardation being only 12.2%. ACTH (Armour) yielded the same type of results as cortisone. Doses of 4–10 mg. per 10 cc. of sea water delayed the rate to the 3–4 blastomere stage by an average of 63%. The delayed effect on the development of plutei with the use of ACTH was also observed, just as in the case of cortisone.

The effect of cortisone on cellular permeability.[1] VALY MENKIN AND MAX PEPPER.

The decrease in capillary permeability in inflammation induced by cortisone is well known (Menkin, 1940, 1942). Previous investigators have reported no striking changes in the appearance of marine eggs exposed to cortisone. Is the rate of penetration of water in the ova of *Arbacia punctulata* decreased by transferring the eggs to hypotonic sea water? The diameter of spherical ova was determined by an ocular micrometer. The eggs were then transferred to 50% sea water. The diameter of the ova was then measured at rapid successive intervals for 20–25 minutes. In each experiment 40–50 ova were measured. The same procedure was repeated with the addition of one mg. of cortisone acetate (Merck) in 10 cc. of sea water. In one observation 2.5 mg. of the corticosteroid was utilized. Overcrowding of ova was avoided in order to bring the suspended cortisone in as close contact with the ova as possible. The preliminary period of exposure to the corticosteroid prior to transfer to 50% sea water ranged from 22 to over 45 minutes. The ova were studied at a magnification of 440 ×. The diameter of the ova exposed to cortisone *per se* tended usually to show a very slight increase in size. Subsequent to their transfer to 50% sea water there followed a decrease in the rate of expansion of the eggs. This, although not invariably, occurred in the first few minutes after transfer. The average decrease in the expansion of the eggs in 6 separate

[1] Aided by a grant from the U. S. Public Health Service, and by a grant from Dr. A. Wander, S.A., Berne, Switzerland.

experiments with one mg. concentration of cortisone acetate was 34%. In the additional experiment involving 2.5 mg. of cortisone, the decrease was 48%. These data are interpreted as an indication that cortisone acetate decreases somewhat, but definitely and consistently, the permeability of sea urchin ova to water.

The adjuvant action of chelating agents in fertilizin agglutination of starfish sperm.[1] CHARLES B. METZ.

Starfish fertilizin ordinarily has no visible effect upon species sperm but in the presence of an "adjuvant" the fertilizin agglutinates the sperm in a specific and striking manner. Animal sera, hen's egg white, peptides and α amino acids were found to be effective adjuvants in earlier studies (Metz, 1945; Metz and Donovan, 1950). Although these agents appeared to act by exposing more receptors (antifertilizin) on the sperm surface, the mechanism of this action has remained obscure. However, it seems likely that the adjuvant effect involves metal binding for the above agents can bind metals. Furthermore, certain other metal binding and chelating agents have now been shown to have adjuvant action. Finally, the adjuvant action of chelating agents is inhibited by metal cations.

Chelating agents which show adjuvant action include Versene (approximately 0.01 *M* in sea water; tested on: *Asterias forbesii, Luidia clathrata, Patiria miniata*) and 8-hydroxyquinoline (saturated solution in sea water; *Asterias, Luidia*). However, citrate (0.5 *M*) proved to be ineffective. Other effective metal binding or precipitating agents are thioglycolate (0.05 *M* in sea water; *Luidia, Patiria*) and hydrogen sulfide (saturated solution in sea water; *Luidia*). The adjuvant action of Versene is inhibited by Ni^{++} (*Patiria*); the similar action of 8-hydroxyquinoline is inhibited by Co^{++} and Cu^{++} but not by Ca^{++} (0.5 *M*).

These observations in conjunction with the earlier studies suggest that most of the sperm surface receptors (antifertilizin) are blocked by metal cations and are unavailable for combination with fertilizin. In the presence of the appropriate metal binding agent (adjuvant) the metal cations are removed, thereby freeing the receptors for combination with fertilizin. Under appropriate conditions this combination results in agglutination. This view further implies that the sperm surface receptors have metal binding properties. Sulfhydryl groups, α amino acid or other chelating structures may be essential parts of these receptors.

Reversible clotting in Spirogyra. W. J. V. OSTERHOUT.

When cells of *Spirogyra* are placed in 0.1 per cent duponol solution (chiefly sodium lauryl sulfate) which kills the cell, the chlorophyll leaves the chloroplasts and a green solution fills the cell. The green color diffuses out when the cell is cut in water.

When the intact cells are transferred from the duponol to a dilute lugol solution (KI + I_2) the contents of the cell become dark and shrink to a stiff gel forming a cylindrical mass in the center surrounded by a colorless liquid. When the cell is cut in water the stiff gel comes out and maintains its form.

The shrinkage in the intact cell is not reversible when the cells are transferred from the lugol solution to water or to buffer solutions at various pH values but it is reversible when the cells are transferred back to 0.1 per cent duponol. The stiff gel disappears and the cell is again filled with the green solution.

This may be repeated many times.

If iodine is omitted the shrinkage occurs more slowly but it is reversible in duponol.

If cells are placed first in lugol solution and then in duponol there is little or no shrinkage.

Shrinkage occurs in lugol solution with cells that have been left standing in 0.1 per cent duponol from a few minutes to several weeks.

If cells are first exposed to duponol and then placed in five per cent trichloroacetic acid the shrinkage occurs until a rounded green mass containing colorless areas is formed. Small green rounded bodies appear in the rest of the cell. When the cells are transferred to duponol these structures disappear and the cell again shows a uniform green color.

[1] This study was aided by grants from the National Institutes of Health, U. S. Public Health Service and the American Cancer Society.

A study of the growth of oysters under different ecological conditions in Great Pond. MADELENE E. PIERCE AND J. T. CONOVER.

A study of the seasonal growth of oysters under different ecological conditions was begun in Great Pond, Falmouth, Massachusetts, in the summer of 1953.

Great Pond, a tidal estuary 2.5 miles long and 0.5 miles wide, is composed of one large main basin which divides at its upper end into two narrow arms. One arm receives the Coonamessett River; the other arm, known as Perch Pond, receives only a small creek. At the outlet the main basin opens into Nantucket Sound; hence the salinity gradient ranges from fresh to 31 parts per thousand through a longitudinal section of the estuary.

On July 1, cages of two-year old oysters were placed at six stations chosen to include widely varying environmental conditions. The investigations were planned to cover at least one complete year, but during the winter most of the cages were stolen or their labels destroyed. Therefore the growing period on which measurements could be recorded was limited to an interval of 10 weeks, July 1–Sept. 9. Measurements of the length, width, depth, and volume of 240 oysters were made at the beginning and at the end of this period.

During the year monthly measurements of important physical and chemical properties included temperature, salinity, dissolved oxygen, and pH. During the limited survey period, at the six stations chosen, bottom temperatures varied from 22°–25° C. At one of two stations representing extremes in seasonal salinity ranges, located one mile from the Sound, bottom salinities varied between 29.7–30.5 parts per thousand, or 0.8; whereas at the other station, in Perch Pond, the range was 12.2–26.3 parts per thousand, or 14.1. The seasonal oxygen utilization and CO_2 production were particularly high at the brackish station in Perch Pond where the maximum increase in growth of the experimental oysters was recorded. The salinity gradient appeared to be the only suggestive limiting factor at some stations. Tidal water exchange was very limited at the station of maximum growth.

Oysters placed on muddy substrate died or showed little growth. Of those placed on sand, oysters living in the lower and more widely varying salinities showed the most growth.

With cages attached to heavier anchors and less conspicuous floats, these oyster experiments are being continued in the summer of 1954 along with productivity studies being made by members of the Woods Hole Oceanographic Institute.

Rhythms of O_2-consumption in the earthworm, Lumbricus terrestris. C. L. RALPH.

Oxygen-consumption of the earthworm, *Lumbricus terrestris*, was measured continuously for three consecutive semilunar periods, April 27 to June 9. The twenty-nine day period from May 12 to June 9, which was more fully analyzed, showed a bimodal diurnal variation in the rate of O_2-consumption, with maxima at 5 A.M. and 7 P.M. and with minima at 1 P.M. and 11 P.M. An analysis to demonstrate any primary lunar cycle for the same period showed one to be present which exhibited a major maximum at 11 P.M. and a minor maximum at 12 noon, with minima at 9 A.M. and 3 P.M. The last two semilunar periods were analyzed for diurnal variation and it was found that each showed essentially a bimodal pattern; the highest peak of the May 12 to 26 period occurred in the A.M. and the highest peak of the May 26 to June 9 period in the P.M. Analysis of the three semilunar periods separately for a primary lunar component showed in each case the presence of a rhythm possessing 12.4 hour cycles. In the first semilunar period maxima were nearly coincidental with lunar zenith and nadir; in the second period the maxima preceded these lunar times by 3 or 4 hours; and in the third period, the form of the cycles continued to become altered and the minima now were at the approximate times of lunar zenith and nadir.

Chromosome behavior during conjugation of mating types I and II of variety 1 of Tetrahymena. CHARLES RAY, JR.[1]

Chromosomal behavior of Elliott's mating types I and II of variety 1 of *Tetrahymena pyriformis* was studied by means of aceto-orcein-gelatin squash and by De Lamater's basic fuchsin staining schedule. The mating types have a haploid chromosome number of five.

[1]Aided by grants from The University Center in Georgia and from The McCandless Fund of Emory University.

Meiosis takes place during the first two pre-zygotic divisions of the micronucleus. Pairing of the homologous chromosomes is accomplished just prior to or during the elongation of the "crescent" in prophase of the first division. Five bivalent chromosomes at diplotene are present as the crescent shortens from its maximum length. One or more chiasmata are present per bivalent. The five pairs of chromosomes condense and become oriented on the spindle. Five chromosomes are seen going to each pole at first anaphase. The second meiotic division follows quickly. Five chromosomes are seen at metaphase of second division. After the completion of the second division only one of the four micronuclear products divides again; the other three nuclei gradually degenerate. The functional nucleus is spindle shaped during prophase and located anteriorly, at the attachment membrane. The haploid number of five chromosomes is seen at third division. Anaphase is oriented antero-posteriorly in the conjugants and stretches nearly the length of the animal. Ten chromosomes are seen during the first postzygotic division of the synkaryon.

Histochemical studies of mesenteric structures. EVELYN KIVY ROSENBERG AND JOSEPH CASCARANO.

Neotetrazolium (NT), a water-soluble salt which becomes a purple, insoluble formazan upon reduction, has been used extensively within recent years as a histochemical tool for the demonstration of dehydrogenase activity in the living cell. Much work has been reported on the reaction of parenchymatous tissues such as kidney, adrenal, liver with this tetrazolium salt but little, if any, on small blood vessels, lymphatics, and peripheral nerves.

By the use of an osmotic, balanced medium we have been able to demonstrate endogenously (in living, viable tissues), a reaction between neotetrazolium and such blood vessels, lymphatics, and nerves in the mouse and rat. The medium finally decided upon is a 0.5% NT solution containing a modified Krebs formula made up of NaCl, KCl, $CaCl_2$, $MgSO_4$, $NaHCO_3$, KH_2PO_4 and buffered to a pH of 7.4 with phosphate. The tissues (*i.e.*, mesenteries or slices of organs) are incubated either aerobically or anaerobically for about three hours at 37° C. The metabolic reduction of NT is halted by immersion in 10% neutral formalin.

We have been able to distinguish readily arterioles, capillaries and venules, lymphatics (20–100 μ), and extremely delicate nerve fibers in mesenteries, as well as in organs; *i.e.*, bladder, tongue, uterus and fallopian tubes. The appearance of formazan in the blood vessels is one of discrete, granular deposition in the cytoplasm not only of the endothelium of blood vessels but also the muscular elements. The laying down of reduced tetrazolium in walls of lymphatics and nerve endings is also particulate. These structures may be readily distinguished from connective tissue fibers since the latter do not reduce tetrazolium.

With this tool, we are at present exploring methods for distinguishing changes in specific enzyme systems in different tissues following x-radiation.

The effect of various levels of x-irradiation on the gametes and early embryos of Fundulus heteroclitus. ROBERTS RUGH.[1]

The gametes of *Fundulus heteroclitus* are quite radioresistant as determined by the effect on subsequent development of the embryo; 25,000 r x-rays to the unfertilized eggs caused a wide range of abnormalities from quite normal to absence of forebrain. Eggs exposed to 100,000 r allowed some embryos to develop quite normally. These were probably haploids, developing parthenogenetically. 200,000 r to the eggs prevented their fertilization. Such an exposure to the eggs did prevent their fertilization. Such an exposure to the sperm did not prevent their motility nor their ability to fertilize normal eggs. While none of the resulting embryos from 200,000 r sperm ever hatched, many developed pulsating hearts connected with a circulatory system completely devoid of corpuscles. The sperm appeared better able to effect normal development following high levels of x-irradiation than did the eggs.

When the one- and two-cell stages were x-rayed to 300 r there was a stunting of development but no gross abnormalities, while 1,000 r caused complete suppression of the anterior neural differentiation. From 2 to 8 cells the maximum tolerable dose which allowed normal

[1] The laboratory facilities for this study were provided by the Office of Naval Research.

development was 500 r but at stage 11 (expanding blastula) even 1,000 r had no appreciable effect on development and it required 3,000 r to produce anterior end teratologies. Thus, the early fertilization and cleavage stages were the most radiosensitive.

In embryos which survived radiation exposure for some time the primary breakdown seemed to be in the circulatory system, followed by stasis in development affecting the central nervous and other systems. Pulsating hearts developed and were functional for some days, even without circulating cells. Cyclopia, complete absence of eyes and forebrain occurred while the more posterior structures appeared to be quite normal. Apparently the normal morphological movements at the time of gastrulation were affected and this caused structural abnormalities and final death of the embryos.

Antibiotics in relation to the survival of Spisula ova. VICTOR SCHECHTER.

The unfertilized egg cells of the clam, *Spisula solidissima*, were stored in sea water containing antibiotics in concentration ranging from 0.5 to 100 units per cc. While controls survived for a maximum of 32 hours, the ova in antibiotic-treated sea water endured for over 90 hours, and were then responsive to sperm. Dihydrostreptomycin and Chloromycetin were most effective. Aureomycin, Penicillin, Bacitracin, Polymyxin and Terramycin followed, in the order given. In some cases the maximum effect was obtained in the maximum concentration used, e.g., Chloromycetin; while with some of the other antibiotics the maximum effect was obtained at lower concentrations. With Aureomycin, for example, the maximum effect was obtained in a concentration of 25 units per cc. Cleavage could occur but was generally retarded in time, and to an extent inhibited. It was evident that the action of antibiotics consisted of more than a single effect, in that it involved the medium in which the eggs were contained, and the eggs themselves. These two effects were antagonistic in their action.

The effect of antibiotics upon Spisula sperm. VICTER SCHECHTER.

Spisula sperm placed in sea water containing antibiotics in concentration of 100 units per cc. generally lost their motility. In the case of Aureomycin and Polymyxin the effect was reversible. It was irreversible with Magnamycin. In Chloromycetin no impairment of motility was detected. Unlike the effect upon survival of Spisula ova, the action of antibiotics upon sperm may be taken to be directly upon the cells themselves, in its entirety, and unrelated to other biotic factors in the medium.

Evidence for active transport of water in Fundulus embryos. D. R. SHANKLIN.

The adult *Fundulus* exists in sea waters whose osmotic strength varies from two to four times that of the fish itself, the euryhaline state. Copeland (1948) has demonstrated the existence of an extra-renal osmoregulatory mechanism whose net effect is maintenance of such gradients in the face of frequently rapid changes. Developing *Fundulus* embryos are also subject to this euryhaline state. In the laboratory they exist and develop in the extremes of distilled water and at salt concentrations twice that of sea water. Evidence for water movement is best obtained by studies with deuterium oxide. The effect of osmotic change on salt balance, however, affords evidence for water movements. Comparison of salt balance in media whose colligative property coefficients are constant with those whose salt concentrations are constant, but heterosmotic, should reflect the order of osmotic strengths. This in fact does not occur. Since all such media are hypotonic to balance, dilution should occur, but in some instances concentration occurs. In those in which dilution does occur, the order is not related to the osmotic scale. These phenomena are more closely related to changes in the water gradient, as determined by densiometric studies—which property does not quantitatively parallel the tonicity. Comparisons of heterosmotic and anisohydric states with normals in presence of glycolytic inhibitor fluoride shows a connection between energy mechanisms and water movement. In presence of fluoride there is egression of water with the osmotic gradient, and ingression against the gradient in the absence of fluoride. These responses permit the embryo to maintain an osmotic pressure 20–25% of the surrounding medium.

The action of trypsin on Arbacia and Echinarachnius eggs in homologous and cross fertilization. ALBERT TYLER AND CHARLES B. METZ.

Trypsin treatment dissolves the gelatinous coat (fertilizin) of unfertilized sea urchin eggs (Tyler and Fox, 1940); it inhibits formation of the fertilization membrane (Kunitz, 1932) and renders the eggs more readily cross fertilizable (Hultin, 1948; Bohus Jensen, 1953). These effects initiated the present study.

Arbacia eggs were treated with 0.05% crystalline trypsin for periods of ½ to 3 hours and aliquots inseminated with serial dilutions of *Echinarachnius* sperm. The concentration of sperm required to yield equal percentages of fertilization in treated and control eggs was then compared. This revealed that trypsin-treated eggs required less than $\frac{1}{27}$ the amount of sperm necessary to obtain comparable fertilization of control eggs. Similarly in the reciprocal test (*Arbacia* sperm × *Echinarachnius* eggs) treated eggs required less than ⅒ as much sperm as controls. The experiments also showed that fertilizability of trypsin-treated eggs by *homologous* sperm was markedly reduced. Treated eggs (*Arbacia*) required 50 × the amount of sperm necessary for the controls. Fertilization and cleavage also occurred in the trypsin solution. *Arbacia* eggs with or without trypsin treatment failed to cross fertilize with *Asterias* sperm. This result suggests that trypsin does not extend cross fertilizability to new combinations but rather facilitates crosses that can occur independently.

Since trypsin obviously removes most of the fertilizin (gelatinous coat) from the eggs, it seemed desirable to determine whether or not some remains as part of the surface. It was found that the addition of antifertilizin (which agglutinates control eggs) inhibits fertilization of both trypsin-treated and control eggs and may agglutinate trypsin-treated eggs. This suggests that at least some fertilizin remains on the surface of trypsin-treated eggs.

Unlike *Strongylocentrotus* and many other species, *Arbacia* sperm following treatment with fertilizin does not suffer a marked loss in capacity to fertilize normal eggs. Since trypsin renders *Arbacia* eggs less readily fertilizable, such eggs appeared to provide a more sensitive test for an effect of *Arbacia* fertilizin on the fertilizing power of homologous sperm. This proved to be correct, for the concentration of fertilizin-treated sperm required to fertilize trypsin-treated eggs was 100 × that of the untreated sperm. This result is interpreted as a decrease in available receptor groups on both the egg (fertilizin) and sperm (antifertilizin).

Loss of radioactivity from tissue sections during histological processing. W. S. VINCENT.

Intracellular distribution of certain substances which will incorporate radioisotopes is possible through the use of radioautograph techniques. Semi-quantitative estimates of the specific activity of certain compounds have been made through the use of enzymatic digestion of tissue sections which have been so labelled, and the counting of reduced silver grains in the emulsion before and after digestion. The extracting effects of aqueous solutions on P^{32}-containing compounds in sections of Amblystoma larvae exposed to 100 μc of P^{32} are given below. The data are expressed as per cent of counts in the section before removal of the paraffin which remain after the indicated treatment. Removal of paraffin: 92; hydration (through alcohols to water): 80; RNAase (two hours, 37°): 25; RNAase control (H_2O): 56; *N* HCl (14 minutes, 60°): 35; HCl controls (H_2O): 66. A similar pattern of loss of radioactivity from starfish oocytes has also been demonstrated.

The nature of the radiophosphorus-containing materials which are soluble in the aqueous solutions has not yet been demonstrated. These data suggest that considerable caution is required in quantitative interpretation of radioautographs.

Induction of a persistent modification in the daily rhythm of O_2-consumption in fiddler crabs.[1] H. M. WEBB, M. F. BENNETT AND F. A. BROWN, JR.

Fiddler crabs of two species, *Uca pugnax* and *Uca pugilator*, were subjected to three cycles of illumination by night and darkness by day. At the end of this time, and again at the con-

[1] This work was supported in part by contracts NONR-09703 and NONR 122803 with the Office of Naval Research.

clusion of the experiment, it was ascertained that the daily rhythm of color change was inverted. The form of the daily rhythm of O_2-consumption of these "reversed" animals under constant conditions was compared with that of controls for two consecutive fifteen-day periods. It was found that the ratio of the average rate of O_2-utilization for the hours between 5 and 10 A.M. to the average rate for the 5 to 10 P.M. hours was about 35% higher in the light-reversed animals than in the controls. This is consistent with the view generally held that the hormone normally involved with the darkening of the body is a depressant for O_2-consumption and that O_2-uptake is lowered at the time of its liberation into the blood and elevated when the titre of this hormone is diminished. Though this described modification occurred, the general form of the daily rhythm, with its tendency for maxima to be present at 3 A.M., 9 A.M., and 3 P.M., with the principal minimum during the evening hours, appeared otherwise unaltered by the light treatment.

Variations in x-radiation sensitivity of different stages of growth in Paramecium.[1] RALPH WICHTERMAN.

After specimens from a given clone of *Paramecium caudatum* are irradiated, considerable variation in x-ray susceptibility is seen to occur depending primarily upon the phases and stages of growth of the organisms when environmental and technical factors are maintained fairly constant during and after irradiation.

Nylon syringes of two-cc. capacity were used as irradiation chambers. In a typical experiment, 200 specimens representing one phase of growth were placed in each syringe. Four syringes containing the different stages were irradiated simultaneously with 250 kiloroentgen. After irradiation, specimens were expressed into sterile spot-plates and kept in moist chambers for observation.

The LD 50 of *Paramecium caudatum*, 24 hours, is approximately 340 kr when fully differentiated, well-fed, vigorous specimens are irradiated from a culture which has reached the peak of maximal division rate. Such specimens when irradiated with 250 kr prove to be more radioresistant than vegetative ones from older cultures containing more transparent, thinner, less vigorous animals in which feeding activity and fission rate are greatly reduced. Twenty-four hours after irradiation, 66 per cent of the vigorous, well-fed specimens survived in contrast to 38 per cent of the other type. Forty-eight hours after irradiation, more specimens in each group succumbed resulting in 49 per cent survival in the former and 21 per cent in the latter. Recovery of x-radiation effects is faster in the survivors of the well-fed, vigorous group than in the other even when both groups of survivors are treated in the same manner.

Based upon these and other experiments, attention is called to the fact that some immobilized paramecia may, for hours, appear dead during the first day after irradiation only to recover after the first 24-hour period. More reliable results may be obtained for *Paramecium* and possibly other Protozoa by determining the LD 50 values for a 48-hour period following x-radiation instead of the commonly used 24-hour period. Also, attention is called to the significance of physiologic conditions and stages of growth of the Protozoa to be irradiated.

An antimitotic substance from muscle.[2] WALTER L. WILSON AND L. V. HEILBRUNN.

According to Bullough, muscular exercise tends to inhibit mitosis in the skin of mice. This may be due to the production of an antimitotic substance by contracting muscle.

As a result of the work of Most, frog muscle is known to produce a heparin-like substance as a result of repeated contractions, and accordingly we began our experiments with muscles of the frog. These experiments are complicated by the fact that in order to use marine eggs for test objects, the salt solution bathing the frog muscles has to be concentrated so that the final tonicity is equal to that of sea water. Accordingly we turned to lobster muscle, using the

[1] Aided by a grant from the Committee on Research, Temple University and performed under Contract NR 135-263 between the Office of Naval Research, Department of the Navy and Temple University.

[2] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

eggs of the worm Chaetopterus to test for antimitotic activity. The tail muscles of lobsters were stimulated electrically. After the muscles, following repeated stimulation, were no longer able to contract, they were cut out of the animal, placed into sea water, and teased apart. After a half hour, the sea water which had been in contact with the muscles was found to be rich in a substance which prevented division of the eggs. Control, unstimulated muscles also give off this substance but in much lower concentration. The antimitotic substance from the fatigued muscles keeps the protoplasm in a fluid state—that is to say, it prevents the mitotic gelation. It is a heat-stable substance and resists boiling. Further studies on its chemical properties are planned.

Inhibition of intermedin-induced darkening of frog skin (Rana pipiens) by tetrazolium salt. PAUL A. WRIGHT.

An interesting physiological property of triphenyltetrazolium chloride (TTC) is its ability to inhibit darkening (dispersion of melanophoral pigment) of isolated frog skin as induced by intermedin. A solution of 0.1% (0.003 *M*) TTC in Ringer's fluid totally prevents the darkening response to an optimal dosage of intermedin (20 units); 0.001 *M* solution is at first inhibitory (30 minutes) and then permits partial darkening; 0.0001 *M* TTC is ineffective. The salt appears to affect the responsiveness of the melanophores and does not merely inactivate the hormone, since a 0.003 *M* solution is just as effective in inhibiting 40 or 400 units of intermedin as it is 2 or 20. The inhibition is immediate and the formation of formazan appears to have no part in the reaction; in fact, photoreduced TTC and freshly prepared solutions are equally potent in preventing darkening. Succinate (0.001 *M*, pH 7.4) did not protect the skin against the action of tetrazolium. There is some indication that glucose (0.01 *M*) can partially release the inhibition imposed by TTC, but levulose and galactose had none of this ability.

TTC did not alter the blanching pattern, which follows darkening, in either Ringer's fluid or solutions of epinephrine, but skins which had been maximally darkened with intermedin returned promptly to the blanched state when TTC was added, even in the presence of an optimal dosage of intermedin (20 units). Addition of TTC at any point during an intermedin-induced darkening causes the melanophores to cease pigment dispersion and begin at once the process of melanin concentration. Further work is in progress to elucidate the mode of action of tetrazolium salts.

Native protein structure and a tetrahedral motif.[1] DOROTHY WRINCH.

Crystal data for many globular proteins direct attention to some striking properties including a spread over all 11 possible classes, cases of pseudo-symmetry, and twins and intergrowths related to the crystal class. The structures of the silicates etc. suggest that these properties may be a consequence of a motif with a tetrahedral quality in the skeletons of protein molecules. This we find in the five atom motif, N—(H—)C_α(—C_β)C, which may be playing for protein molecules a part formalistically similar to that played by the SiO_2 motif in cristobalite, the common feature being the setting of motifs to their fellows with reference to a single set of cubic axes. Picturing such motifs assembled with symmetry 231, R-groups could be inserted with symmetry 231 or 22 or 3 or 2 or 1. Assuming mutual affinities of such structures when apposed in parallel, with or without one-quarter turns about cube edges and one-sixth turns about cube diagonals, explanations of the properties could be given.

LALOR FELLOWSHIP REPORTS

I. A search for L-spinacine in dogfish liver. II. An investigation of the constituents and function of the hypobranchial gland of certain Muricidae. BRUCE N. AMES.

L-spinacine (imidazo-tetrahydropyridine carboxylic acid) was reported to be present in large amounts in shark (*Acanthias vulgaris*) by Ackermann. This compound is of interest

[1] This work is supported by the ONR under contract with Smith College.

because of its close structural relationship to both histidine and purines. We have synthesized spinacine from histidine by the procedure of Neuberger, and have been able to localize it on paper chromatograms by spraying the paper with diazosulfanilic acid, a reagent for imidazoles. No spinacine could be detected on chromatographing extracts of dogfish (*Squalus acanthias*) liver.

Urocanylcholine (murexine) was found in the hypobranchial gland of *Murex trunculus* and several related Mediterranean snails by Erspamer. It is closely related in structure to acetylcholine and has a pronounced curare-like action. The function of the hypobranchial gland in snails is not understood. We have chromatographed the glands of several local Muricidae in an attempt to correlate the feeding habits of the snails with the presence of urocanylcholine. Extracts of the glands were chromatographed on paper and the urocanylcholine spot eluted and read in the Beckman. In addition to a strong ultra-violet absorption, urocanylcholine may be indicated on chromatograms by a diazosulfanilic acid spray. Thais and Urosalpinx contain large amounts of urocanylcholine in the hypobranchial gland but not in other organs. Thais glands also contain several fluorescent compounds and diazo-reacting spots distinct from murexine, histidine, and urocanic acid. Each species of snail gives a characteristic pattern when a particular organ is chromatographed on paper and the chromatogram is viewed with ultra-violet light. No evidence as to the function of the hypobranchial gland has been obtained.

A study of some proteolytic enzymes during the development of Ilyanassa obsoleta. JACK R. COLLIER.

The hydrolytic activity of three peptidases and three proteinases was measured in the egg and at subsequent stages of development of Ilyanassa. The relative activity of all enzymes studied is expressed as microliters of 0.05 *N* HCl necessary to titrate the free carboxyl groups which were released by the enzymatic hydrolysis.

The relative activity of glycylglycine dipeptidase and aminotripeptidase of the Ilyanassa egg was equivalent to 0.11 and 0.22 microliters of 0.05 *N* HCl per egg, respectively. By the fifth day of development (at 19 degrees C.), at which time a rapid growth of the organ primordia begins, the activity of each of these peptidases had increased by about 55 per cent; by the tenth day, when the veliger larva is fully developed, the glycylglycine dipeptidase activity was about 700 per cent greater than the activity found in the egg. Alanyl-l-histidine dipeptidase activity could not be detected in the egg or at any subsequent time during development.

The hydrolytic activity of cathepsin II was not detected at any time prior to the fifth day of development. The activity of cathepsin II in the five-day-old embryo was equivalent to 0.05 microliters of 0.05 *N* HCl per embryo and by the sixth day of development a two-fold increase had occurred. This increase in catheptic activity continued during the remaining four days of development and by the tenth day the cathepsin activity was equivalent to 0.36 microliters of 0.05 *N* HCl. The hydrolytic activity of pepsin was not detected in the egg or in the four-day-old embryo; however, the pepsin activity of the veliger larva was equivalent to 0.56 microliters of 0.05 *N* HCl per larva. The trypsin activity of the veliger was equivalent to 0.13 microliters of 0.05 *N* HCl per larva.

The intracellular localization of the alanylglycine dipeptidase activity in the egg of Ilyanassa obsoleta. JACK R. COLLIER.

The Ilyanassa egg was fragmented by centrifuging in gum arabic for 10 minutes at a centrifugal force of about 2,000 times gravity. The centrifugal fragment contained all of the large yolk platelets and a small amount of hyaline protoplasm, whereas the centripetal fragment consisted of an oil cap and most of the hyaline protoplasm. The centripetal half represented 50.4 per cent of the total volume of the egg and the centrifugal fragment 49.6 per cent. The percentage distribution of the alanylglycine dipeptidase activity was 69.6 per cent in the centripetal fragment and 21.2 per cent in the centrifugal fragment.

The volume of the yolk platelets in the Ilyanassa egg was measured by homogenizing the egg and packing the platelets in a capillary tube with subsequent measurement of the volume of the capillary occupied by the yolk platelets. From these measurements it was found that the yolk occupied 33.4 per cent of the total volume of the egg. The volume of the oil cap was determined by calculating the volume of the spherical segment which it occupied in the centrifuged egg and was found to be 10.5 per cent of the total volume. Thus, the hyaline protoplasm (plus its finer inclusions) was found to occupy 56.1 per cent of the total volume of the egg.

Considering that the centrifugal fragment contained all of the yolk platelets plus some hyaline protoplasm, and that it represented 49.6 per cent of the total volume of the egg the percentage of the hyaline protoplasm in the centrifugal fragment was found to be 16.2 by subtracting the volume of the yolk contained in the egg from the volume of the centrifugal fragment. The percentage of hyaline protoplasm in the centrifugal fragment is in close agreement with the percentage of the dipeptidase activity in the centrifugal fragment. Thus, it seems probable that the alanylglycine dipeptidase activity is contained in the hyaline protoplasm and/or its finer inclusions.

Attempt at isolation of desoxyribonucleoprotein from pneumococci. H. EPHRUSSI-TAYLOR.

Although several types of desoxyribonucleoproteins have been isolated from higher organisms, no evidence has ever been obtained demonstrating the existence in bacteria of desoxyribonucleic acid in a protein-bound form. In collaboration with Dr. Jay Barton, an attempt has been made to isolate from pneumococcus a desoxyribonucleoprotein, employing the technique described by Mazia and Bernstein. Employing distilled water extraction of frozen and thawed bacteria of a streptomycin-resistant strain of pneumococcus, it has been possible to isolate a material which is endowed with transforming activity (conversion of streptomycin-sensitive pneumococci into resistant ones). Characterization of the extracted material is as yet incomplete. Analyses show the extracts to contain appreciable amounts of protein, ribonucleic acid and desoxyribonucleic acid, but the extent to which the components are bound to each other remains to be determined. No material having the solubility of nucleohistone was found. Quantitative studies of transforming activity of extracts strongly suggest that the transforming factor is present in bound or aggregated form, since activity increases 100–1000 fold upon dilution. No inhibitory substances could be detected, which otherwise might have accounted for the low activity of undiluted extracts.

Fine structure in the oocyte nuclear membrane. JOSEPH G. GALL.

Nuclear membranes from isolated oocyte nuclei of the starfish *Henricia* were spread flat on plastic "Formvar" films, fixed in 1% osmium tetroxide, dried, and observed with the electron microscope. The membrane consists of a thin continuous sheet with raised annuli scattered on its cytoplasmic side. The annuli are roughly 1000 Å in diameter with a center about 400 Å across. In thin sections of osmium-fixed oocytes the continuous sheet appears as a fine line about 150 Å thick. The annuli are not easily distinguished from the cytoplasm in transverse sections of the nuclear membrane, but are apparent in areas cut tangentially. Annuli of similar dimensions have been seen in sectioned oocytes of the annelid *Chaetopterus*, and in spread nuclear membranes from oocytes of the frog *Rana*, the newt *Triturus*, and an unidentified minnow. These observations suggest that the nuclear membranes of widely divergent forms may be constructed on a common plan.

The effects of various agents have been tested on unfixed nuclear membranes of *Triturus*. Some solutions appeared to digest the membrane (pepsin, trypsin), while others had little effect on its fine structure (distilled water, various concentrations of sodium chloride up to 1.0 *M*, phosphate buffer at pH 7.0, equal parts of ether and alcohol, and solutions containing calcium or from which this ion had been removed by the chelating agent "Versene"). The nuclear membrane is therefore composed, at least in part, of relatively insoluble protein.

A further study of isometric mechanical responses of the anterior byssus retractor muscle of Mytilus edulis to electrical stimuli and mechanical stretch. WILLIAM H. JOHNSON.

Winton has shown that following direct current stimuli, the anterior byssus retractor muscle of *Mytilus edulis* relaxes very slowly and shows an increase of tensile "viscosity" which can be reversed by a burst of alternating current or short D.C. pulses. The long-lasting D.C. response is cathodal, as shown by Fletcher. Thus, in the present work, a Taylor triangular electrode was used in an attempt to produce as large a cathode as possible at the muscle surface. It was found that a) if the electrode is made cathodal, the response to single pulses was large, if anodal, the response is small; b) a typical D.C. response is obtained at pulse durations as short as 20 msec.; c) the response becomes greater as the pulse duration is increased; d) these responses will sum, until, at high repetition rates, a Winton "tetanic" response is obtained. Quick stretches applied to the muscle showed the muscle to be more plastic following "tetanic" stimuli than following D.C. stimuli, as found by Winton. Even at muscle lengths below rest length, the stiffness increases following D.C. stimuli. This increase is similar to, but smaller than, the increase in stiffness seen during iodoacetate rigor. The "unlocking" or "plasticizing" action of the tetanus takes place during a short interval following the cessation of the stimulus. The hypothesis is advanced that these visco-elastic changes may be produced by variations in intrafibrillar concentrations of ATP, similar to the effects found by Weber using glycerinated psoas fibers. In this muscle, the stiffness can be increased with little concomitant contraction by adjusting the repetition rate of short D.C. stimulating pulses; thus the visco-elastic change might be a major physiological response in such holding muscles.

Some observations on bioluminescences. BERNARD L. STREHLER (with kind assistance of R. Cahn and M. Owens).

Several bioluminescent systems have been examined with respect to general and more specific properties. The chemiluminescence of green plants has been studied with respect to the decay curves of this luminescence during the early period after the illuminating flash. In addition, about 30 species of marine and land plants including brown, red and green algae, conifers, and a variety of monocots and dicots, have been examined for the occurrence and intensity of this luminescence. All plants examined exhibited this luminescence.

The major effort was centered on Mnemiopsis bioluminescence. Many prior observations have been confirmed. Among the newer observations are the following: Stable luminous powders can be prepared by low temperature lyophylization; chemiluminescence can be elicited from cell suspensions by a great variety of physical and chemical agents including mechanical stimuli, electrical shock, detergents, alcohol, freezing, etc. No luciferin-luciferase reaction has thus far been demonstrated. Factors necessary for bacterial or firefly luminescence were ineffective in this system. With the supply of stable powders obtained this summer it is hoped that the biochemical investigations can be carried further during the coming winter.

Large amounts of isolated luminous material from ctenophores have been extracted with organic solvents in an attempt to obtain the pigments responsible for bioluminescence and for the photoinactivation of the luminescence of the intact ctenophore or cell suspensions obtained from it.

Finally, with the hypothesis in mind that light might trigger an internal chemiluminescence which would act as an amplifier in visual receptors, squid and Fundulus eyes were examined with a phosphoroscope-quantum counter combination. No delayed luminescence was observed, indicating that the hypothesis is probably incorrect.

P^{32} incorporation into starfish oocyte nucleoli. W. S. VINCENT.

The incorporation of P^{32} into various fractions of the starfish oocyte nucleolus has been studied by direct analysis of isolated nucleoli and by autoradiography. P^{32} enters the nucleolus at a linear rate over a nine hour period. The relative specific activities of nucleolar fractions are as follows: whole nucleolus, 1.0; acid-soluble P, 3.0; RNAP, 0.5; protein P, 0.2. The

relative specific activity of the whole egg P and cytoplasmic RNAP were 1.0 and 0.5, respectively. The dilute acid-soluble fraction contains non-dialysable nucleotides of relatively high specific activity. Semi-quantitative grain counts of autoradiograms of nucleoli in tissue sections indicate that the RNA of *in situ* nucleoli has a specific activity of 100 to 1000 times that of the cytoplasmic RNA. Ultraviolet absorption measurements on isolated and *in situ* nucleoli have shown that about 10% of the ribonuclease-removable absorption is lost during isolation. Preliminary studies of autoradiographs of aliquots of egg homogenates taken during various intervals of the isolation procedure suggest that considerable radioactivity is lost from the nucleoli during the early steps of isolation.

These data suggest that the starfish nucleolus contains two types of RNA. One type is loosely bound and metabolically labile, as is indicated by the concentration of radioactivity in the material lost during isolation of the nucleolus. The other type incorporates P^{32} quite slowly, and is tightly bound to the nucleolar structure.

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